



New Principles for Targeting Cancer - a Rational Small Molecule Approach

Rønnest, Mads Holger

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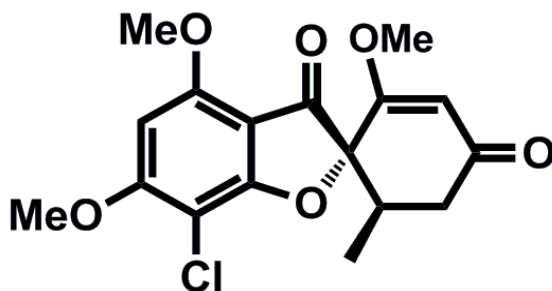
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New principles for targeting cancer - a rational small molecule approach



Mads Holger Rønnest
PhD Thesis
June 2011

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New principles for targeting cancer - a rational small molecule approach

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This PhD thesis was prepared by

Mads Holger Rønnest

Supervisors

Assoc. Prof. Thomas Ostenfeld Larsen	Department of Systems Biology, DTU
Assoc. Prof. Mads Hartvig Clausen	Department of Chemistry, DTU

Department of Systems Biology
Center for Microbial Biotechnology (CMB)
Technical University of Denmark
Søltofts Plads, building 223
DK-2800 Kgs. Lyngby
Denmark

www.bio.dtu.dk

Tel: (+45) 45 25 25 25

Fax: (+45) 45 88 41 48

E-mail: info@bio.dtu.dk

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Preface

This thesis is submitted to the Technical University of Denmark in partial fulfillment of the requirement for the Degree of Doctor of Philosophy in Chemistry. The work was carried out between February 2008 and June 2011, at the Center for Microbial Biotechnology at the Department of Systems Biology and the Department of Chemistry under the supervision of Associate Professor Thomas Ostenfeld Larsen and Associate Professor Mads Hartvig Clausen. January and February 2010 was spent at Deutsches Krebsforschungszentrum (DKFZ), University of Heidelberg, Germany under the supervision of Professor Alwin Krämer, MD. The project was funded by the Danish Research Council (ref. 274-07-0561).

I would like to thank my two supervisors Thomas Ostenfeld Larsen and Mads Hartvig Clausen for three years of incredibly exciting work. You have both been enthusiastic throughout the project and have been an inexhaustible source of ideas for current and future projects, thank you for that. You have also listened when I have told you that I would need another three years to perform all those experiments, for that I am equally thankful. If the rest of my work life proves equally enjoyable, I will be very privileged.

A thank also goes to Alwin Krämer, for letting me work in his research group during my stay in Heidelberg. I learned so incredibly much about biology in general and centrosomal clustering in particular during those two months. I would also like to thank Blanka Leber for not only tutoring me at work but also for opening her and Armin's home to me. A thank to Simon Anderhub for the hard work he has done in the lab and for swift answers to all the questions I have sent his way. Also thank to Dr. Marc-Steffen Raab, MD for his work with GF15 and GF61 and his contribution to the cooperation in general. I will definitely miss the biannual gatherings, filled with important discussions about the future of the project, but also with sufficient time to go to dinner, have a few beers and talk about life in general.

I have received ample amount of help from a number of people: Lisette and Kir have been a great help for both preparing raw extracts and bringing up fungi from the collection, on top of that they have been great fun to work with and their door has always been figuratively open, even if knocking was mostly answered with a NO! :-). Jesper, Kristian and in particular Hanne have been an indispensable help with running the machine park in 221, thank you for that. I

would also like to thank my office mates in 221, Maria and Jesper for some great years. The office has never been in shortage of professional or personal advice. I would also like to thank the rest of 221 for a great work environment and for the many talks around the coffee machine during the years.

I would like to thank Charlotte and Anne for running those many many NMR experiments and for general hall talks. Also thank to Brian for the help during my PhD and in particular for a number of rotations and IR spectra. I would like to thank Palle and Hélène for the time at Mads' group. I had the pleasure of sharing a lab with Mathias and although I never knew where my acetone bottle was, or for that matter anything else in the lab, it was a time with lots of organic chemistry talk as well as a fair share of friendly banter. Also a thank to the rest of the people in 201 for making the last three years enjoyable.

Last I would like thank my family for the support during the last three years. That goes for my mom and Niels who took good care of Nanna, Iben and Asger when I worked nights and weekends during the last few months. The same goes for Børge, Hanne, Ellen, Peter and Mette who all visited in the weekends, enabling me to work even more. Thanks to Børge for moral support on the phone when things got rough. Thanks to Andreas and Lea for moving excel data around and for moral support. I am also thankful to Iben and Asger, whom at days of work despair could blow all worries away with a few smiles. Most of all though I am grateful for having such a wonderful wife, she has been an absolutely invaluable help throughout the PhD. Thanks for keeping the house pretty much by yourself for a long time towards the end and thanks for all the professional help that you gave during the three years.

Abstract

Cancer is the leading cause of death in the developed world and research is ongoing both towards curing the disease and understanding the disease on a cellular level. As most cancer cells proliferate very fast, mitosis has often been the target of anti-cancer agents. However, this does not afford cancer specific drugs as healthy cells are also affected, albeit to a lower extent. An ideal anti-cancer drug would target cancer cells specifically, thus obviating side effects.

A difference between healthy and cancerous cells that is currently being explored is the supernumerary centrosomes present in most cancer cells. Centrosomes are the microtubule organizing centers during mitosis and here, one centrosome is situated at each pole of the mitotic spindle. During anaphase, each sister chromosome will be pulled towards the centrosomes in bipolar mitosis. Supernumerary centrosomes enable the cancer cells to undergo multipolar mitosis, introducing chromosomal instability, which would make it unlikely to produce viable progeny. The cancer cells circumvent multipolar mitosis by a mechanism called centrosomal clustering, where any numbers of centrosomes are gathered in two clusters, thus making bipolar mitosis possible. A small molecule that could disrupt centrosomal clustering and force the cells to undergo multipolar mitosis might possess the ability to affect cancer cells specifically.

Prior to the work in this PhD thesis, the fungal metabolite griseofulvin (**1**) was identified as an anti-cancer compound in a bio-guided screen of fungal raw extracts in a phenotypic based assay. The assay was designed to identify compounds that forced cancer cells to undergo multipolar mitosis and thus apoptosis, using the cancer cell line SCC114 (squamous cell carcinoma cells). In this work two structure activity relationship (SAR) studies have been completed. The first investigated the anti-cancer SAR of griseofulvin analogs in the phenotypic based assay and the other a growth inhibition SAR against the two dermatophytes *Trichophyton mentagrophytes* and *T. rubrum* as well as toxicity against the cancer cell line MDA231 (human breast adenocarcinoma).

The studies found a good correlation between the anti-cancer phenotypic IC₅₀ values and the cytotoxicity values ($R^2 = 0.70$). General trends showed that alterations of positions 4, 5 and 6 of griseofulvin lower the activity, while elongation of the 2' position increases the activity. Bulkier substituents increased the activity even further with the 2' benzyloxy analog (**2**) being the most potent. The

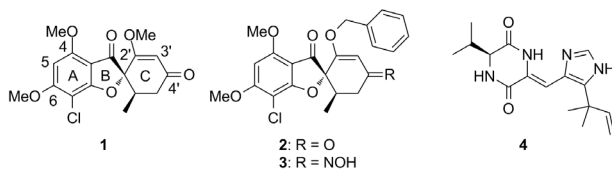


Figure 1: The structures of griseofulvin (**1**), 2'-benzyloxy-2'-demethoxy-griseofulvin (**2**), the 4' oxime (**3**) of the latter and aurantiamine (**4**)

introduction of an oxime at the 4' position increase the potency slightly in both assays but did not show any cumulative effect with the 2' benzyloxy group (**3**). After extensive synthesis of 2' analogs, the benzyloxy (**2**) and benzyloxy oxime (**3**) analogs were still the best lead compounds.

It is not possible to distinguish between the two aryl methoxy groups in griseofulvin (**1**) by NMR. To unequivocally prove the position of the two possible phenols and thus the methoxy groups, the two phenols were synthesized and distinguished by single crystal X-ray analysis. In ^1H NMR, the 4-methoxy group resonates upfield from the 6-methoxy group. A quantitative yield was obtained for the synthesis of the 4 phenol by treatment of **1** with MgI_2 , while the 6 phenol was obtained in 29% yield by treatment of **1** with LiI in pyridine. (+)-Geodin shares the same structural core as griseofulvin (**1**) and it was isolated from *Aspergillus terreus* to be tested in the phenotype based assay. The compound showed no induction of multipolar mitoses but was twice as potent in the cytotoxicity assay compared to **1**. The unknown absolute structure of (+)-Geodin was assigned by single crystal X-ray analysis.

The compound aurantiamine (**4**) was isolated from *Penicillium cavernicola* and found to induce multipolarity in SCC114 cancer cells with an IC_{50} of 40 ± 4 μM . This was further investigated by fluorescence-activated cell sorting (FACS) analysis showing M phase arrest in SCC114 cells and an increase in the G_0 population indicating apoptosis. Further indications of apoptosis was observed using Western blotting for the p85 fragment of poly (ADP-ribose) polymerase (PARP), a downstream target of activated caspase 3.

In addition to the above mentioned studies, a novel technique for the introduction of radioactive iodide to aryl groups was developed. This was used in scintigraphic *in vivo* imaging to assess bio-distribution and half-life of a griseofulvin analog. Treating an aryl trimethylsilyl precursor with 2 equiv. of thallium(III)trifluoroacetate in 30% TFA in MeCN for 2 minutes followed by addition of sodium ^{125}I or ^{131}I affords the radio labeled compound in over 95% radioactive yield. This method is applicable for both ortho, meta and para TMS aryl precursors stable in TFA/MeCN (3:7).

Dansk Resumé

Kræft forårsager de fleste dødsfald i den udviklede del af verdenen og kræftforskning har derfor høj prioritet. Forskningen fokuserer både på at finde midler, der kan helbrede personer ramt af kræft, men også på at forstå hvordan kræft udvikles. De fleste lægemidler i dag er ikke specifikt rettet mod kræftceller men rammer cellernes mitose. Da kræftceller deler sig meget hurtigere end de fleste andre celler i et menneske, bliver kræftcellerne dog hårdere ramt end de raske celler. Ikke desto mindre ønskes et ideelt lægemiddel, der dræber kræftceller og kun kræftceller.

En måde hvorpå at finde denne slags lægemidler er at se på hvilke forskelle, der er på raske celler og kræft celler. En sådan forskel er de unormalt mange centrosomer, der findes i kræftceller. I raske celler er der to centrosomer under mitosen, hvor de virker som microtubuli organiserende centre. Under bipolar mitose sørger de for delingen af datterkromosomerne ud i de to datter celler. Kræftcellerne med mange centrosomer har muligheden for at gennemgå en multipolar mitose, hvilket højst sandsynligt ville resultere i ikke levedygtige datter celler. Kræftcellerne undgår dette ved hjælp af centrosomal klyngedannelse, hvor alle centrosomer bliver klumpet sammen i to klynger, der så kan fungere som to poler som i en regulær bipolar mitose. Et molekyle, der kan inhibere denne centrosomale klyngedannelse, kan potentielt virke specifikt mod kræftceller.

Griseofulvin (**1**, se Figur 2) blev, forud for dette arbejde, identificeret som et anti-kræft stof i en screening af svampe rækstrakter i et phenotype baseret assay. Dette assay er designet til at identificere molekyler, der kan få kræftceller til at gennemgå multipolar mitose. Der er blevet gennemført to struktur-aktivitet sammenhængsundersøgelser i dette arbejde. Det første omhandlede anti-kræft egenskaberne af griseofulvin analoger i det phenotype baserede assay, mens der i det andet blev testet analoger mod to patogene svampe *Trichophyton mentagrophytes* og *T. rubrum* samt en cancer cellelinje.

Der var en god korrelation mellem de IC₅₀ værdier, der blev fundet i det phenotype baserede assay, og toksisitets-assay'et mod kræftcellelinjen ($R^2 = 0.70$). Ud fra data kunne det konkluderes, at ændringer i positionerne 4, 5 og 6 generelt resulterede i en lavere aktivitet, mens forlængelse af 2' position øgede aktiviteten. Større grupper øgede aktiviteten yderligere med en 2' benzyloxy gruppe som den mest aktive. En oxim i 4' positionen øgede aktiviteten i begge assays, men en

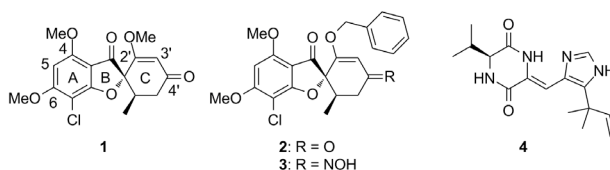


Figure 2: Strukturerne af griseofulvin (**1**), 2'-benzyloxy-2'-demethoxy-griseofulvin (**2**), 4' oximen (**3**) af den foregående samt aurantiamine (**4**)

analog med både en 2' benzyloxy gruppe samt en 4' oxim viste ikke akkumuleret aktivitet. Efter grundig undersøgelse af 2' positionens betydning kunne det konkluderes at 2'-benzyloxy analogen samt versionen med en 4'-oxim stadig er de mest potente.

De to methoxy-grupper i griseofulvin kan ikke skelnes ved hjælp af NMR. For at bevise positionen af de to methoxy-grupper blev phenolerne af griseofulvin syntetiseret og positionen af disse blev bestemt ved hjælp af enkeltkrystal røntgenanalyse. På denne måde kunne positionen af methoxy grupperne også bestemmes, og 4-methoxy gruppen er upfield fra 6-methoxy gruppe. 4-phenol analogen blev syntetiseret i kvantitativt udbytte med MgI_2 . 6-phenolen blev syntetiseret med LiI i pyridin i 29% udbytte.

(+)-Geodin har samme grundstruktur som **1** og blev derfor isoleret fra *Aspergillus terreus* for at blive testet. Det viste sig at (+)-geodin ikke inducerede multipolære mitoser i SCC114 kræftceller, dog var (+)-geodin dobbelt så aktiv i toksicitets assayet i forhold til griseofulvin (**1**). (+)-Geodin's absolutte struktur var ukendt men blev bestemt ved hjælp af enkeltkrystal røntgenanalyse til at være *R* ved spirocentret. Aurantiamine (**4**) blev isoleret fra *Penicillium cavernicola* og inducerede multipolar mitose i SCC114 kræftceller med en IC_{50} på $40 \pm 4 \mu\text{M}$. Indvirkningen af **4** på SCC114 celler blev også undersøgt med fluorescens-aktiveret celle-sortering (FACS), og der blev observeret en ophobning af celler i M fase og en øget G_0 population, hvilket kan indikere apoptose. Ved hjælp af Western blotting efter p85 fragmentet af poly (ADP-ribose) polymerase (PARP) blev det yderligere påvist, at cellerne lavede apoptose.

En ny teknik til at mærke aromatiske stoffer med radioaktivt iod blev udviklet. Behandling af en aromatisk trimethylsilyl (TMS) forbindelse med 2 ækvivalenter $\text{Ti}(\text{OOCF}_3)_3$ i MeCN/TFA (7:3) efterfulgt af radioaktivt kalium iodid (^{125}I eller ^{131}I) førte til det radioaktivt mærkede stof i over 95% udbytte. Denne metode er brugbar for både aromatiske *ortho*-, *meta*- og *para*-aryl TMS grupper så længe de er stabile i MeCN/TFA (7:3).

List of Publications

Mads H. Rønneest, Blanka Rebacz, Lene Markworth, Anette H. Terp, Thomas O. Larsen, Alwin Krämer and Mads H. Clausen. *Synthesis and Structure-Activity Relationship of Griseofulvin Analogues as Inhibitors of Centrosomal Clustering in Cancer Cells*. J. Med. Chem. **2009**, 52, 3342-3347.

Alwin Krämer, Blanka Rebacz, Mads H. Clausen, Thomas O. Larsen, Mads H. Rønneest, Kasper Worm-Leonhard. *Griseofulvin Analogues for the Treatment of Cancer by Inhibition of Centrosomal Clustering*. WO2010072770, **2010**.

Mads H. Rønneest, Pernille Harris, Charlotte H. Gotfredsen, Thomas O. Larsen and Mads H. Clausen. *Synthesis and single crystal X-ray analysis of two griseofulvin metabolites*. Tetrahedron Lett. **2010**, 51, 5881-5882.

Mads H. Rønneest, Morten T. Nielsen, Blanka Leber, Uffe H. Mortensen, Alwin Krämer, Mads H. Clausen, Thomas O. Larsen and Pernille Harris. *(+)-Geodin from Aspergillus terreus*. Acta. Crystallogr. C. **2011**. 67, o125-o128.

Mads H. Rønneest, Marc S. Raab, Simon Anderhub, Sven Boesen, Alwin Krämer, Thomas O. Larsen, Mads H. Clausen. *Disparate SAR Data from Griseofulvin Analogs Tested Against the Dermatophytes Trichophyton mentagrophytes, T. rubrum and MDA231 Cancer Cells*. Submitted to J. Med. Chem.

Marc S. Raab, Iris Breitzkreutz, Mads H. Rønneest, Blanka Leber, Thomas O. Larsen, Ludmila Wagner, Simon Anderhub, Gleb Konotop, Patrick J. Hayden, Klaus Podar, Johannes Fruehauf, Felix Nissen, Walter Mier, Uwe Haberkorn, Anthony D. Ho, Hartmut Goldschmidt, Kenneth C. Anderson, Mads H. Clausen, and Alwin Krämer. *GF-15, a novel inhibitor of centrosomal clustering, suppresses multiple myeloma growth in vitro and in vivo*. Submitted to Blood.

Non peer reviewed publication:

Mads H. Rønneest, Kasper Worm-Leonhard, Mads H. Clausen, Thomas O. Larsen. *Svampenaturstoffer som grundskelet for nye typer af lægemidler mod kræft*. Dansk Kemi. **2009**, 90, 6-8.

Thesis Outline

The work for this thesis entitled "New principles for targeting cancer - a rational small molecule approach" is based on the cooperation between the Center for Microbial Biotechnology at the Department of Systems Biology and the Department of Chemistry both at the Technical University of Denmark and the German Cancer Research Center at the University of Heidelberg, Germany. The thesis includes work performed at all three locations, in the fields of natural products chemistry, organic chemistry and microbiology.

The thesis consists of nine chapters. Chapter 1 is a short introduction to cancer, centrosomal clustering, and natural products as bioactive compounds. Chapter 2 is a review of the chemistry of griseofulvin, intended for submission to Chemical Reviews upon further work. Chapter 3 is a published paper concerning an anti-cancer structure-activity relationship study of griseofulvin analogs in a phenotypical assay. Chapter 4 is a manuscript concerning the structure-activity relationship study of griseofulvin analogs against two dermatophytes as well as a cancer cell line in two growth inhibition assays, which has been submitted to the Journal of Medicinal Chemistry.

Chapter 5 is a published paper distinguishing between the two aryl methoxy groups of griseofulvin. Chapter 6 presents the work performed on the development of a radiolabeling technique of aryl trimethylsilyl precursors. Chapter 7 is a published paper concerning the isolation of (+)-geodin and the determination of the absolute structure of (+)-geodin. Chapter 8 presents, the work concerning the isolation of aurantiamine and the work performed with this natural product at the German Cancer Research Center (DKFZ), University of Heidelberg. Chapter 9 is the final conclusion of the entire work.

A paper with the title "GF-15, a novel inhibitor of centrosomal clustering, suppresses multiple myeloma growth *in vitro* and *in vivo*", which has been submitted to Blood is found in Appendix A. Co-author statements for all articles and drafts in the thesis can be found in Appendix B. Supporting information of articles and drafts can be seen in Appendix C-G. To restrain the number of pages in the Appendix most of the supporting information have been modified to only contain the ^1H and ^{13}C NMR spectra of synthesized/isolated compounds. Full supporting information for a given article can be obtained at the appropriate journal homepage.

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In developed countries the single highest cause of death for man is cancer and in developing countries it is the second highest cause.¹ Therefore the attention to and funding for cancer research is massive and it has accelerated in the late 20th century with for example the National Cancer Act of 1971 in the United States.² The focus in cancer research is both on treating the disease³ as well as understanding what goes on at the cell biology level.⁴

So what is cancer? In short it is cells that proliferate abnormally and are able to invade other tissue. It involves the transformation from normal to fully malignant cells through a number of mutations (see Figure 1.1). One mutation or combinations of mutations afford the cells with one or more of the malignant phenotypes required for the cells to develop into being fully malignant. Some phenotypes are: insensitivity to antigrowth signals, mitogen-independent growth, evasion of apoptosis, immortalization, sustained angiogenesis and metastasis.^{5,6,7} It's well known that smoking increases the risk of lung cancer⁸ and another example of an exogenic factor that is life style related is obesity, which increases the risk of renal cancer.⁹ However, factors which people have no or little influence on, like ethnicity and socioeconomic status^{10,11,12} also have a correlation with the risk of developing cancer.

Cancer can be treated by chemotherapy,¹³ radiation therapy¹⁴ or surgery¹⁵ and often involves a combination of these treatments.¹⁶ For chemotherapy a popular drug target has been mitosis and although not truly cancer specific, anti-mitotic drugs are expected to affect malignant cells to a greater extent than healthy cells. After all, uncontrolled cell growth and proliferation is a defining phenotype of most malignant cells and thus the effect from disrupting mitosis should be greater on the malignant cells than on healthy, slower proliferating cells.¹⁷ However, some healthy cells also proliferate fast and the side effects of chemotherapy often stem from the drugs effect on these cell types. An example is hair bulb cells that are often affected by cytotoxic anti-cancer drugs resulting in hair loss (alopecia).¹⁸

Anti-mitotic agents often target tubulin,¹⁹ with two major groups, those that stabilize microtubules (e.g. Taxol®,²⁰ epothilones²¹) and those that destabilize tubulin (e.g. the *vinca* alkaloids²²). In both cases microtubule dynamics are greatly reduced, affecting mitosis²³ and resulting in cellular M phase arrest followed by apoptosis.²⁴

The ideal anti-cancer drug would target malignant cells and malignant cells only and thus potentially obviate the side effects of chemotherapy. In order to achieve this, differences between malignant cells and healthy cells must be explored and described. A difference in phenotype that is currently being explored is the fact that centrosome amplification^{25,26,27,28} is a common feature in human

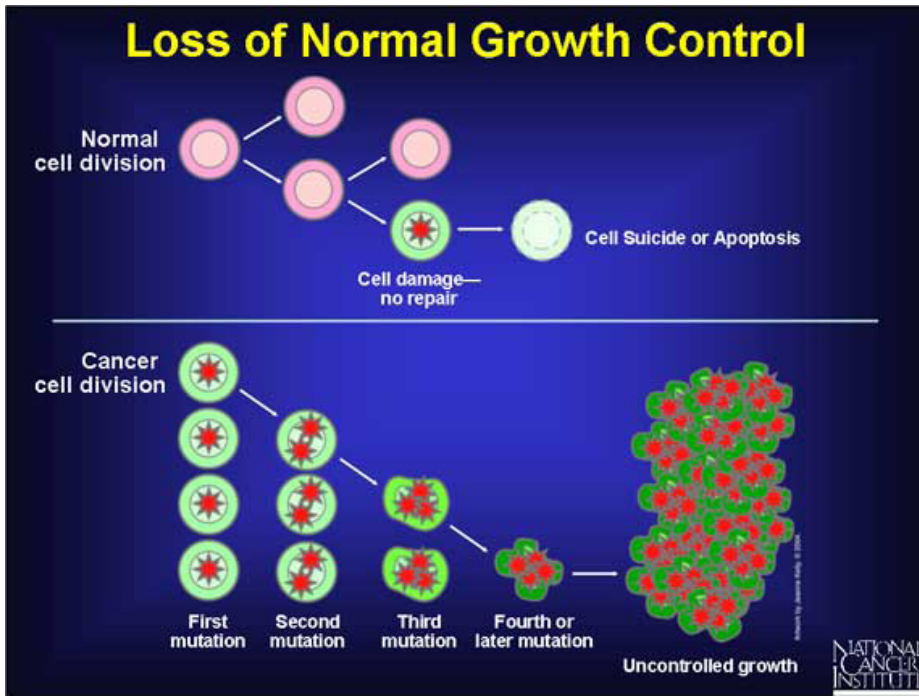


Figure 1.1: On top normal cells are depicted with one daughter cell showing DNA damage. This cell will undergo apoptosis because of the many tumor suppressor mechanisms present in the cell. On the bottom more cells with DNA damage are shown, which for some reason are not forced to undergo apoptosis, possibly because the damage has affected some of the tumor suppressor genes. This cell population can then acquire more mutations over time and end up with enough cancer phenotypic characteristics to become fully malignant (called the multistep process). Artwork originally created for the National Cancer Institute. Reprinted with permission of the artist, Jeanne Kelly. Copyright 2011.

cancer cells. If not for a phenomenon called centrosomal clustering,²⁹ cancer cells would undergo multipolar mitoses resulting in aneuploid daughter cells, which are determined to undergo apoptosis. A schematic overview is depicted in Figure 1.2, where a cell with supernumerary centrosomes undergoes bipolar mitosis with the majority of the progeny undergoing apoptosis. There is an odd chance of a daughter cell surviving the multipolar mitosis and regaining chromosomal stability by centrosomal clustering. This cell is now able to undergo bipolar mitosis and to proliferate. A small molecule that inhibits centrosomal clustering would force this initial survivor to continuous multipolar mitosis resulting in unviable progeny at some point. Such a small molecule would potentially offer a specific treatment of cancer.³⁰

1.1 Centrosomal Clustering

A centrosome (see Figure 1.3) consists of two centrioles surrounded by an electron dense matrix of protein aggregates, referred to as the pericentriolar material (PCM).³² The centrosomes are microtubule organizing centers and are essential for correct segregation of chromosomes in diploid cells during bipolar mitosis.³³ During mitosis (see Figure 1.4) of healthy cells the two centrosomes³⁴ are situated at each pole of the mitotic spindle, and in anaphase one copy of each duplicated chromosome will be pulled to each pole. This results in two daughter cells each with one centrosome and one identical set of DNA after cytokinesis. During the next cell cycle the centrosome and DNA will be duplicated exactly once.

Whereas normal cells contain two centrosomes during mitosis³⁴ it is common that malignant cells contain supernumerary centrosomes.^{25,26,27,28} Having more than two centrosomes could facilitate multipolar mitosis, with subsequent aberrant chromosome segregation and the development of aneuploid daughter cells, which most likely will undergo apoptosis.^{7,31}

Centrosome amplification can happen through a number of pathways (See Figure 1.5): faulty regulation of centrosome synthesis³⁵, abrupted mitosis³⁶, fusion of two cells³⁷, fragmentation of centrosomes into smaller fractions that are still able to function as microtubule organizing centers^{38,39}, over-expression of certain pericentriolar material proteins producing a-centriolar microtubule organizing centers³⁵, *de novo* synthesis of centrosomes^{40,41} or decoupled DNA and centrosome cycles.⁴² Regardless of the path to supernumerary centrosomes, it evokes chromosomal instability upon the cancer cell, and thus a property to acquire more mutations.

There are currently two models for loss and/or gain of chromosomes for cells with supernumerary centrosomes. A regular multipolar mitosis with three or more poles affording aneuploid daughter cells (see Figure 1.6). In this case it is most likely that tripolar mitosis affords aneuploid but viable daughter cells.⁴³ It is also possible for cells with extra centrosomes to align in a pseudo bipolar fashion (see Figure 1.6). In this case one centrosome could fail to align with the bipolar axis while still being able to nucleate microtubules and capture chromosomes at their kinetochores.⁷ During anaphase the misaligned centrosome would pull chromosomes out of the bipolar axis and one daughter cell would receive extra chromosomes while the other would have lost them (see Figure 1.6).

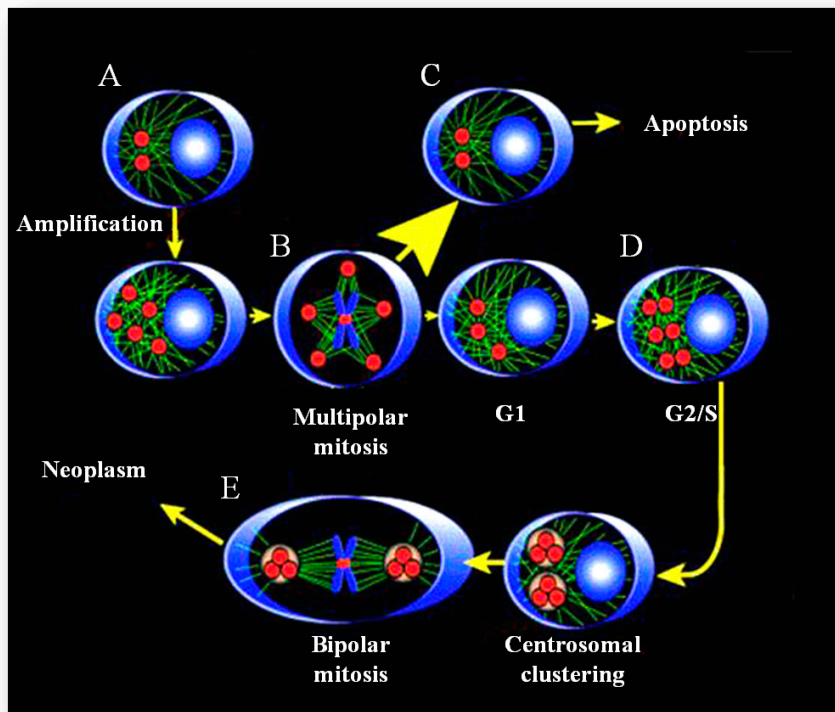


Figure 1.2: (A) The number of centrosomes in a cancer cell is amplified, which can occur through multiple possible pathways (see Figure 1.5). (B) The cell now undergoes multipolar mitosis with most progeny receiving insufficient DNA to survive, leading to apoptosis (C), while it is possible that one cell receives enough DNA to survive and re-enter the cell cycle (D). The cell needs to regain chromosomal stability to be viable and this is achieved by centrosomal clustering, which enables the cancer cell to undergo bipolar mitosis despite its supernumerary centrosomes (E). Reprinted by permission from Macmillan Publishers Ltd: *Oncogene*³¹, copyright 2001.

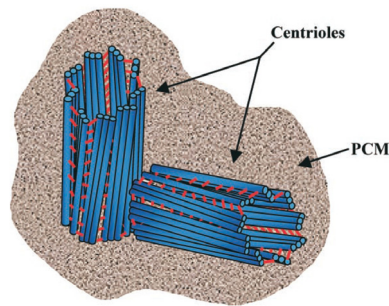


Figure 1.3: A simple presentation of the centrosome. A pair of centrioles perpendicular to each other embedded in a cloud of pericentriolar material (PCM). The centrosomes function as the main microtubule organizing center in the cell. Reprinted by permission from Macmillan Publishers Ltd: Oncogene³⁵, copyright 2002.

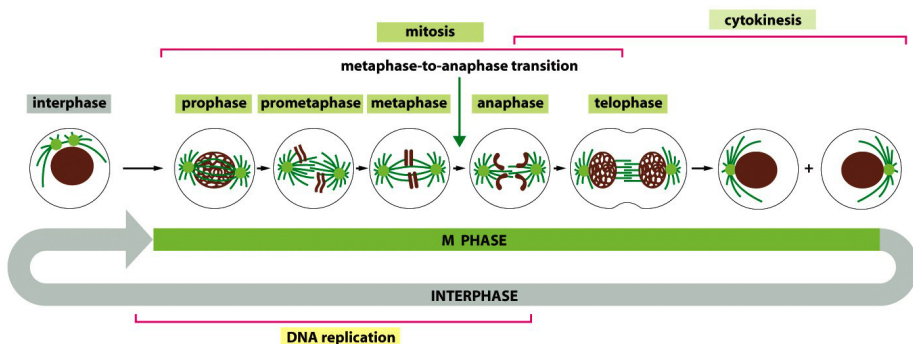


Figure 17-3 Molecular Biology of the Cell 5/e (© Garland Science 2008)

Figure 1.4: The cell cycle of a healthy cell is depicted. DNA and centrosome replication occur in S phase (not shown), during interphase. In prophase the unordered DNA is folded into sister chromosomes and in metaphase the mitotic spindle is assembled. Here the chromosomes are aligned in between the two centrosomes at each pole. During anaphase each sister chromosome is pulled towards a centrosome followed by telophase, where the contractile ring initiates cytokinesis. After completed cytokinesis two identical daughter cells have been formed, which can now proliferate further. Copyright © 2008 from Molecular Biology of the Cell by Bruce Alberts et al. Reproduced by permission of Garland Science/Taylor & Francis Books, Inc.

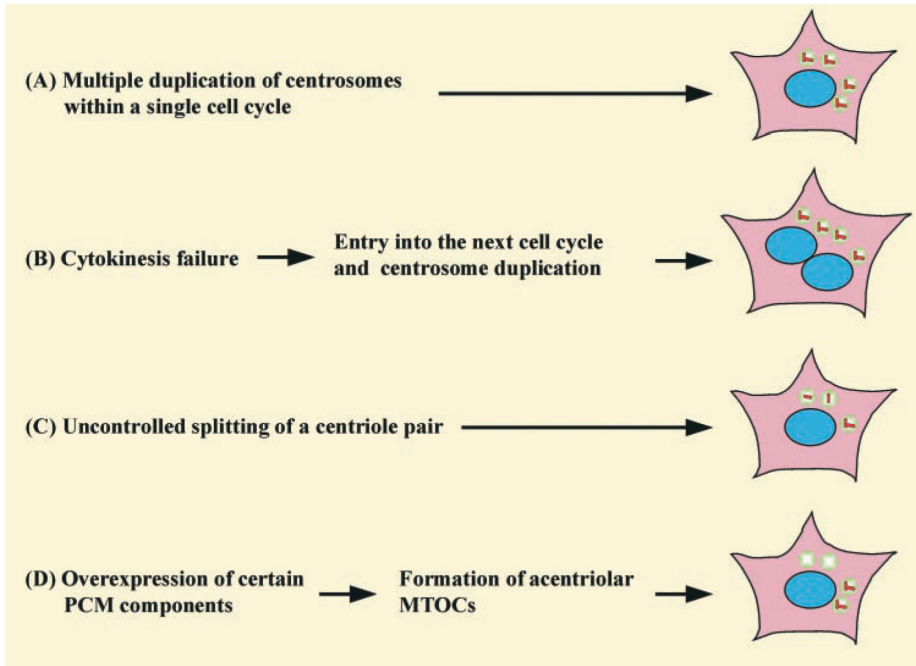


Figure 1.5: Four possible pathways to supernumerary centrosomes. (A) A cell that has synthesized extra copies of centrosomes during a single cell cycle will contain extra centrosomes. (B) Aberrant mitosis will leave the cell with two sets of copied chromosomes and two centrosomes that will be duplicated in the next cell cycle leading to multiple centrosomes in a single cell. (C) If splitting of the two centrioles of one centrosome is unsupervised it could result in more than two functional microtubule organizing centers. (D) Increased production of pericentriolar material due to over expression could also result in more than two functional microtubule organizing centers. Reprinted by permission from Macmillan Publishers Ltd: Oncogene³⁵, copyright 2002.

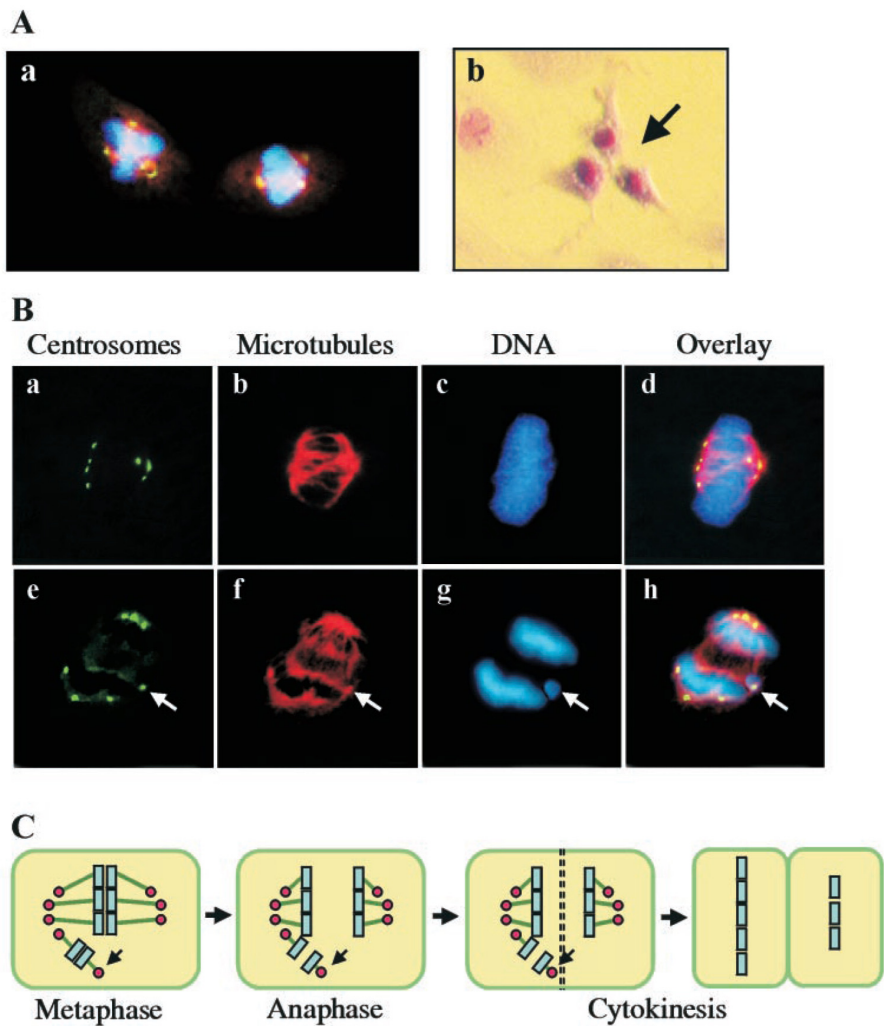


Figure 1.6: Multipolar and pseudo-multipolar mitosis with cells harboring supernumerary centrosomes are pictured. (A) Two tripolar metaphase spindles are shown in (a), centrosomes are yellow, microtubules in red and DNA in blue. (b) Three daughter cells from a tripolar mitosis are seen. These three cells should show significant alterations to their DNA content as compared to the mother cell. (B) A cell with supernumerary centrosomes has aligned in a pseudo bipolar fashion, but with a single centrosome out of alignment. During anaphase, this centrosome will pull chromosomes out of the bipolar axis, which results in aneuploid daughter cells as one will have lost and one gained chromosomes. (C) A schematic presentation of the chromosome loss/gain pathway. Reprinted by permission from Macmillan Publishers Ltd: *Oncogene*³⁵, copyright 2002

In a tripolar mitosis there are still only two copies of DNA and dividing the genes between the three daughter cells would most likely render them all aneuploid leading to apoptosis. It is possible to imagine though, that during the multitude of cell divisions in cancer cells, one daughter cell arising from a multipolar mitosis would receive a sufficient amount of DNA to survive. It is also possible that mutations or genes missing will grant the cell a new cancer phenotype. Continual proliferation of this aneuploid cell is dependent on regaining chromosomal stability, as continual multipolar mitoses would most likely result in apoptosis at some point. An aneuploid daughter cell with supernumerary centrosomes needs to acquire the ability to undergo bipolar mitosis and this can be achieved by centrosomal clustering.²⁹

As cancer cells need to cluster their centrosomes to undergo bipolar mitosis and healthy cells do not, a small molecule that inhibits centrosomal clustering would potentially be specific towards cancer cells. A phenotype based assay to search for such small molecules was established by Prof. Dr. Alwin Krämer MD, and Dr. Blanka Rebacz from DKFZ, Germany.⁴⁴ See section 1.6 for a short description of the assay.

1.2 Natural Products with Anti-cancer Properties

Medicine derived from nature has most likely been used since before civilization started, with the earliest known records from Egypt around 2900 BC.⁴⁵ Today almost 5000 years later, nature is still the major contributor of drugs to mankind. In a period from January 1981 to October 2008, 1024 new anti-cancer drugs were introduced on the market. Of those drugs only 37% were truly synthetic, the rest were in some way inspired by nature.⁴⁶ This indicates that looking for novel bioactive compounds in nature is still a valid strategy.⁴⁷

There is a vast pool from which natural products can be derived, compounds have been isolated from plants,²⁰ insects,⁴⁸ bacteria,⁴⁹ fungi,⁵⁰ and marine organisms⁵¹ (See Figure 1.7 for examples of compounds). In the case of fungi, a raw extract is produced by grinding the fungi and agar in a stomacher with an organic solvent, which upon concentration affords the raw extract. With a raw extract available there are several approaches to the isolation of the natural products, one method is to isolate every novel compounds from a given extract and then possibly test them in various assays afterwards. Another method is the bioguided search for natural products where an assay must be available to test raw extracts. If the target is known, it is possible to do target oriented isolation. A number of raw extracts can then be tested in the assay and hopefully one or more will show activity. With an active raw extract the isolation of the bioactive compound can begin, which is often an iterative process with retesting after each fractionation step. The isolation will most likely be done on a (semi)preparative HPLC system using various normal or reverse-phase chromatography methods. A third option is to use explorative solid phase extraction (E-SPE),⁵² where more information about the chemical properties of the target compound is obtained before isolation starts. This is attained by using four orthogonal solid phase columns, utilizing not only polarity but also charge and size of compounds. This method makes it possible to plan an isolation strategy before making a larger raw extract.

Going through the laborious efforts to make large raw extracts followed by isolation of known compounds is frustrating and can be a potential problem.⁵³ With isolated bioactive natural products counted in the tenths of thousands, natural product chemists face an ever increasing task in distinguishing between novel and known compounds. Early dereplication (see section 1.3)) is vital to natural product chemists and when working with microbial natural products the process can be assisted by e.g. Antibase,⁵⁴ which was developed by Prof. Dr. Hartmut Laatsch. It is a program that enables the scientist to compare physical properties, e.g. exact mass or UV spectra of a target natural product, with compounds already available in the database. This search should indicate whether the active compound is a known or novel compound and thus if isolation of the compound should be completed.

A possible problem for bioactive natural products with sufficient potency to go to clinic trials is availability. If isolation from nature only procures minimal amounts, if fermentation is not viable or if total synthesis is too lengthy or inefficient, then availability becomes a bottleneck. This was the case with Taxol® (**1**), as it was not possible to make this very potent agent available for clinical trials because of its scarce availability. It was not until the isolation of a precursor of Taxol® (**1**) from the needles of *Taxus baccata* that a semisynthetic route made the compound available in larger quantities.⁵⁵

When a bioactive secondary metabolite has been isolated and characterized, a structure-activity relationship study can be performed pending availability. This is done by altering various positions on the parent compound and subsequently test the analogs in the assay used for the bioguided isolation or in other suitable assays. The modifications can be achieved by total synthesis of the parent compound and analogs, or if enough of the parent compound can be isolated by altering directly on the parent compound. This will yield information about the important positions of the compound and improved potency could result in a new lead compound. This lead compound will then be the basis of a new round of optimization.

1.3 Dereplication

The earlier dereplication can be used to determine if an active fraction is due to a novel or known compound, the more work and time can be saved. Running a raw extract through a HPLC-DAD-HRMS systems will afford a lot of information and data, making the tentative assignment of a given compound in the raw extract plausible. This setup will give a general idea of the polarity of a compound, the UV spectrum affords information about possible functional groups as well as the conjugation of the compound, but most importantly the exact mass of a compound will be of great help in the dereplication of a given raw extract.

When using MS to find the exact mass of a compound, correct interpretation of the MS data is pivotal. The assignment of $[M+H]^+$ or $[M-H]^-$ can be complicated by minor co-eluting compounds that ionize better than the target compound. The use of adducts (e.g. $[M+Na]^+$, $[M+H+MeCN]^+$ and/or $[M+NH_4]^+$) are of great assistance to correct assignment of the molecular peak. By recognizing the pattern of these adducts it is possible to assign $[M+H]^+$ or $[M-H]^-$ with

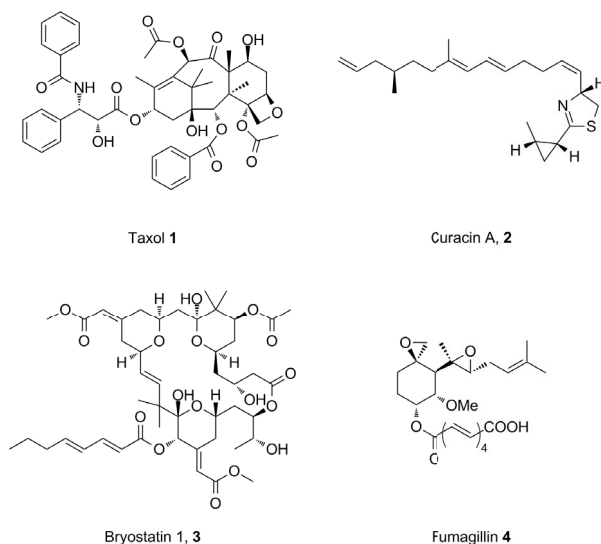


Figure 1.7: Four anti-cancer agents derived from four different natural sources. Taxol® (1) (plant),²⁰ Curacin A (2) (Cyanobacterium),⁵⁶ bryostatin 1^{57,58} (3) (marine symbiot bacteria) and fumagillin (4) (fungi).⁵⁹

confidence.

There are however some limitations to this approach, compounds that do not ionize in either negative or positive mode and does not have a UV chromophore will not be detected, these could in turn be observed using a light scattering detector.⁶⁰

Depending on the accuracy on the MS equipment the observed mass of a compound could be compatible with several molecular formulas. Even if very high accuracy is obtained there are still different compounds with the same molecular formula. Using all information available: polarity, UC spectra, MS-MS, exact mass it is often possible to discriminate between the hits in antibase acquired from the exact mass.

1.4 Secondary Metabolites from Fungi

Secondary metabolites or natural products can be defined as metabolites not normally involved in primary metabolic processes. The discussion about why secondary metabolites are produced at all, ended with the now accepted notion that natural products evolve under the pressure of nature.⁶¹ Secondary metabolites can be divided into different classes depending on their biosynthetic origin, namely polyketides, terpenes and alkaloids. Natural products can be one of these types solely or any mix of these three. A chromatogram of a raw extract from *Aspergillus terreus* can be seen in Figure 1.8, with each peak representing a metabolite.

Secondary metabolites fulfills some role in the life of the fungi producing them,

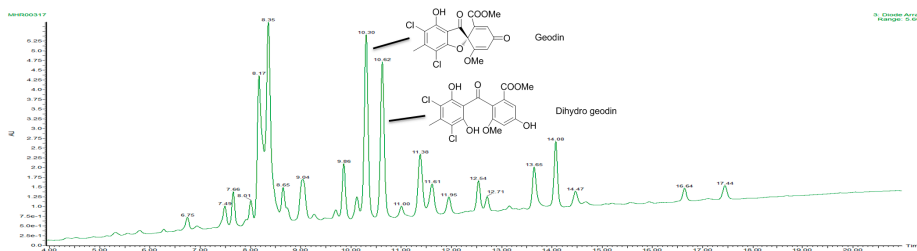


Figure 1.8: HPLC-DAD chromatogram of a raw extract from *Aspergillus terreus*, illustrating peaks corresponding to natural products. The known compounds geodin and dihydro geodin have been assigned.

e.g. by interacting with other microorganisms like bacteria or other fungi. The interaction with bacteria can be extremely helpful for man, as seen when fungi entered the world stage as a source for anti-bacteria agents with the discovery of penicillin.^{62,63} The interaction with other fungi makes it possible that some of these bioactive compounds could have effects on mammalian cells including cancerous cells as both fungi and mammals are eukaryotic; the cell filaments actin and tubulin are for example very conserved in eukaryote cells.⁶⁴ An example of a fungal metabolite which shows both anti-fungal^{65,66} and anti-cancer properties is griseofulvin.^{67,68}

Mycotoxins are bioactive compounds that are toxic to humans, these mycotoxins interacts with humans, mostly through food sources⁶⁹ or fungi infested living quarters.⁷⁰ Some of the more important mycotoxins are the aflatoxins⁷¹ and ochratoxin A.⁷² Aflatoxins are produced by fungi contaminating e.g. maize, which can cause liver cancer in humans consuming the maize.⁷³ Ochratoxin A is found in many consumer products like coffee,⁷⁴ wine⁷⁵ and meat.⁷⁶

1.5 Work flow

Looking for bioactive natural products involves a number of disciplines and processes (see Figure 1.9). The source of the secondary metabolites needs to be handled, the production of a raw extract, testing of the raw extract in a given assay, dereplication to avoid isolating known compounds, isolating a possible novel bioactive compound, structure elucidation and characterizing of the compound. This can then be followed by a structure-activity relationship study of the compound, which could result in a new lead compound.

1.6 Centrosomal Clustering Assay

The assay⁴⁴ was designed to identify small molecules that inhibits centrosomal clustering. The cell line initially used was an oral squamous cell carcinoma cell line labeled SCC114, which was cloned to stably express green fluorescent protein (GFP)- α -tubulin. The cell line contained centrosome amplification in 64.5% of the cells but only 3.6% of mitotic cells harbored multipolar spindles. A large por-

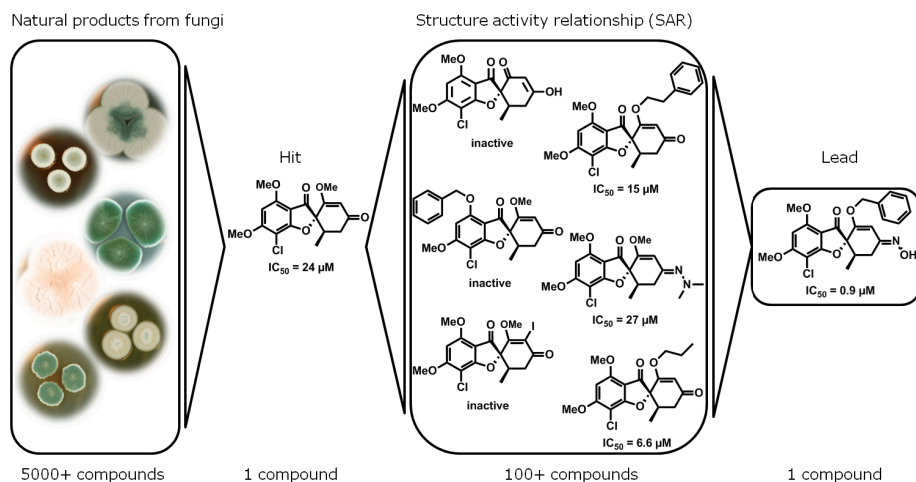


Figure 1.9: Starting from the left is the the source, in this case fungi, of natural products. Narrowing down from the numerous compounds in a screen with raw extract to a hit, in this case griseofulvin. In the case of griseofulvin tentative identification was achieved by dereplication of the initial raw extract. Had it been a novel compound the process of isolation followed by structure elucidation should have been completed. With the active compound characterized it is possible to do a structure-activity relationship study. This can be done by e.g. total synthesis or in the fortunate case of griseofulvin by modifying directly on the commerciable availabe compound. A SAR study could afford better analogs and a new lead compound.

tion of the cells with supernumerary centrosomes thus underwent bipolar mitosis by centrosomal clustering.

For screening purposes the cells were grown to near confluence and then treated with raw extract at different concentrations for 7 h, fixed, and examined by fluorescence microscopy. Three hundred mitotic cells per well were examined, observing if the cells underwent single, bi or multipolar mitosis, with the readout being the percentage of multipolar mitoses. BJ fibroblasts was used as a noncancerous control cell line to investigate the specificity of a given active extract.

The screening procedure was changed around July 2008 to use the untransformed SCC114 cells. Cells were seeded and incubated for 24 h after which raw extract or griseofulvin analogs were added at various concentrations. After fixation the cells were stained with Eg5 antibodies and evaluated by fluorescence microscopy. The number of cells counted per well was lowered to one hundred, easing the laborious manual counting of cells. Around May 2010 the cell line used for the assay was changed as the SCC114 cells had lost their centrosomal amplification. The SCC114 cells were replaced by a human cell line labeled MDA-MB-231 (breast adenocarcinoma), which at present is the cell line used in the assay.

1.7 Griseofulvin

During the initial screening by Assoc. Prof. Thomas Ostenfeld Larsen, Lisette Knoth-Nielsen (technician) and Blanka Leber (PhD. student at the time), the known natural product griseofulvin was tentatively identified as active in three separate raw extracts (*Penicillium berlinense*, IBT 18288 and 19440 and *P. faroense*, IBT 22543). The effect of griseofulvin was further investigated at DKFZ and during my undergraduate work I synthesized three 2' analogs showing increased activity, which was included in the paper concerning the assay.⁴⁴ A large part of the work performed in this work is based on this discovery of griseofulvin in the assay.

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Chemistry of Griseofulvin

Griseofulvin (**1**, see Figure 2.1) was initially isolated from *Penicillium griseofulvum* in 1939 by Oxford et al.¹ and was later isolated from *P. janczewskii* as "Curling Factor" by Brian et al.^{2,3} in 1946. The latter described the curling of hyphae induced by **1**.^{2,3} It was not until 1947 that it was proved that griseofulvin (**1**) and "Curling Factor" was the same compound.^{4,5} The analogous metabolites 7-bromo-7-dechloro-griseofulvin⁶ (**2**, 1954) and dechlorogriseofulvin⁷ (**3**, 1953) were isolated by MacMillan.

The structure of the compound was investigated by means of IR and UV spectroscopy as well as degradation studies.^{8,9,10,11} The correct relative structure for griseofulvin (**1**) was reported in 1952 by Grove et al.¹² followed by a number of papers concerning the chemistry of **1** and synthesis of analogs (1952-1957).^{13,14,15,16,17} The absolute structure of **1** was reported in 1959 by Macmillan¹⁸ and has since been supported by a crystal structure.¹⁹ There are two possible epimers of griseofulvin, position 2 and 6', but through the literature they are both referred to as epigriseofulvin.

The anti-fungal properties of **1** have been known since 1958,²⁰ but recently potency against cancer²¹ and hepatitis C infected cells²² have renewed the interest in this compound making a review of griseofulvin relevant. In the following sections a number of subjects concerning griseofulvin will be described, including total synthesis, analogs obtained by total synthesis and analog synthesis using **1** as starting material.

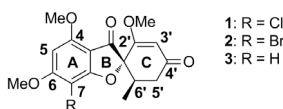
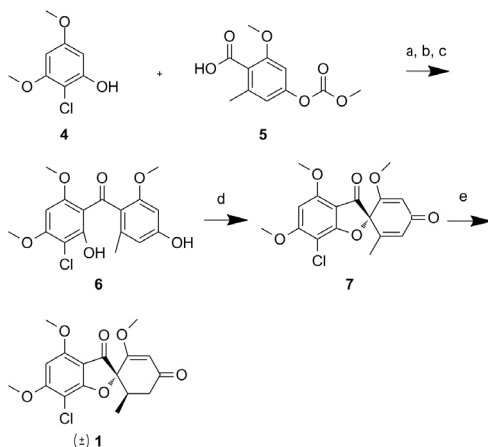


Figure 2.1: griseofulvin (**1**), 7-bromo-7-dechloro-griseofulvin (**2**) and dechlorogriseofulvin (**3**).

2.1 Total Syntheses of Griseofulvin

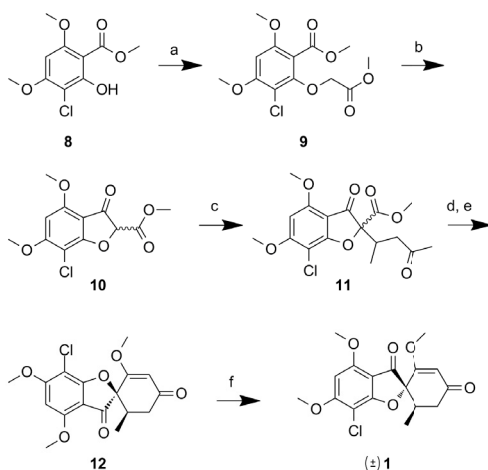
Several different strategies have been utilized in the total synthesis of racemic griseofulvin and the synthesis of the natural product (+)-griseofulvin (**1**) was published in 1990. The first total synthesis was completed by Day et al. (1960)^{23,24} by oxidative cyclization of a benzophenone precursor (**6**, see Scheme 1), a strategy based on earlier pondering on the biosynthesis of **1**.^{25,26} The two arenes 2-chloro-3,5-dimethoxyphenol¹¹ (**4**) and 2-methoxy-4-((methoxycarbonyl)oxy)-6-methylbenzoic acid²⁷ (**5**), with the latter treated with oxalyl chloride to afford the acid chloride were used as starting materials. The benzophenone (**6**) was prepared by Fridel-Craft reaction of **4** and the acid chloride of **5** followed by alkaline treatment. The formation of the spiro center was completed by radical formation using potassium ferricyanide affording (\pm)-dehydrogriseofulvin (**7**). The final step was hydrogenation of **7** with a preformed catalyst of 3% selenium and 5% rhodium on charcoal affording (\pm)-griseofulvin (**1**).



Scheme 1: (a) $(\text{COCl})_2$, benzene; (b) AlCl_3 , nitrobenzene; (c) 4M NaOH, ether, nitrobenzene; (d) Na_2CO_3 , $\text{K}_3\text{Fe}(\text{CN})_6$, H_2O ; (e) 5% Rh/C containing 3% selenium, H_2 , EtOH

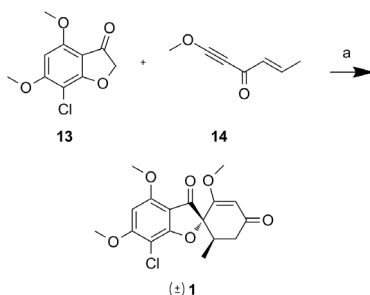
The C ring of griseofulvin (**1**) was established by a Dieckmann cyclization in the total synthesis by von A. Brossi et al. (1960) (see Scheme 2).^{28,29,30} The synthesis of the substituted 3-coumarone **10** starts with **8**, which was treated with methyl 2-bromoacetate to afford **9** setting up the first Dieckman cyclization yielding **10**. The 3-coumarone **11** was synthesized by Michael addition to 3-penten-2-one. A second Dieckmann cyclization followed by treatment with diazomethane afforded 2-epi-griseofulvin (**12**), which was isomerized with sodium methoxide to yield (\pm)-griseofulvin (**1**).

Stork and Tomasz (1962)^{31,32} reported the use of a double Michael addition to establish both stereocenters and the 2' enol ether in a one-pot synthesis (see Scheme 3). The key substrate in this synthesis was an alkoxyethynyl vinyl ketone (**14**) used as a double Michael acceptor. 7-Chloro-4,6-dimethoxy-3-coumaranone (**13**) was used as precursor and reaction with **14** and potassium *t*-butoxide in



Scheme 2: (a) $\text{BrCH}_2\text{COOMe}$, K_2CO_3 , DMF; (b) Na, toluene, 100 °C; (c) 3-penten-2-one, Triton B, MeOH; (d) NaOMe, MeOH; (e) CH_2N_2 , MeOH; (f) NaOMe, MeOH, 80 °C

diglyme/*t*-butyl alcohol afforded (±)-griseofulvin (**1**).



Scheme 3: (a) *t*-BuOK, 2-methoxyethyl ether, *t*-BuOH

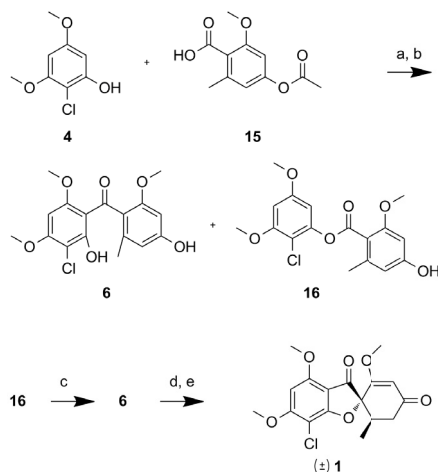
Utilizing a method described earlier for the synthesis of 6'-desmethyl-griseofulvin³³ and 7-dechloro-7-fluoro-griseofulvin,³⁴ Taub et al. (1962)^{35,36} used oxidative cyclization to establish the spiro center of (±)-griseofulvin (**1**) (see Scheme 4). In the early communication the last step was introduction of the chloro substituent, making this a formal total synthesis of **1**.³⁵

The total synthesis as described in the full paper³⁶ prepared the benzophenone (**6**) from two aromatic precursors as by Day et al.^{23,24} The arene (**15**) destined to become ring C of **1** differed slightly as the phenol was protected by an acetyl group and the acid instead of the acid chloride was used. The coupling with **1** was performed with *in situ* formation of the mixed anhydride from trifluoroacetic anhydride affording both **6** and the ester **16**. Transformation of **16** to the desired benzophenone (**6**) was completed by mild hydrolysis followed by a Fries rearrangement.

For the following ring closure to (±)-dehydrogriseofulvin (**7**) a number of successful methods were described. Quantitative yields was obtained using potassium

ferricyanide, lead dioxide in ether/acetone or manganese dioxide in ether/acetone. Hydrogenation to **1** was performed in ethyl acetate using 10% palladium on charcoal as catalyst.

Danishefsky and Walker (1979)³⁷ reported a total synthesis of **1** utilizing a Diels-



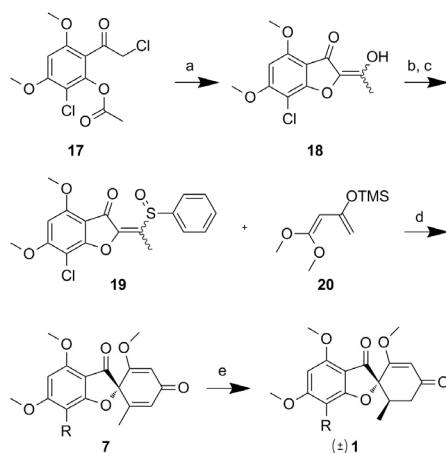
Scheme 4: (a) $(F_3CCO)_2O$; (b) 5% NaOH in aq. MeOH; (c) $TiCl_4$, nitrobenzene; (d) $K_3Fe(CN)_6$; or PbO_2 , ether, acetone; or MnO_2 , ether, acetone; (e) 10% Pd/C, H_2 , EtOAc

Alder cycloaddition strategy (see Scheme 5), an improvement of the total synthesis of (\pm) -2-epigriseofulvin by Danishefsky and Etheredge.³⁸ The dienophile (**19**) was synthesized from **17** through double intramolecular nucleophilic attack affording **18**, followed by treatment with thiophenol and oxidation with *m*-chloroperoxybenzoic acid. The dienophile reacted with the diene (**20**) in toluene at 100–135 °C affording (\pm) -dehydrogriseofulvin (**7**), which was hydrogenated to (\pm) -griseofulvin (**1**).

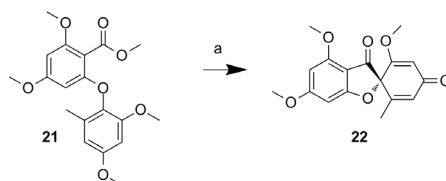
A total synthesis of (\pm) -dechloro-dehydro-griseofulvin (**22**) was completed by Sargent (1980)³⁹ (see Scheme 6). Treating the precursor **21** with dry hydrogen chloride and excess titanium tetrachloride in dichloromethane yielded **22** through oxidative cyclization.

An improvement of the method by Stork and Thomas using double Michael addition was published by Yamato et al. (1990)⁴⁰ (see Scheme 7). The 3-coumarone (**13**) was reacted with an altered Michael acceptor (**23**) yielding **24** and through treatment with activated alumina (\pm) -2'-demethoxy-2'-methylthio-griseofulvin (**25**) was formed. Displacement of the thio ether was achieved by oxidizing sulfur with *m*-chloroperoxybenzoic acid followed by treatment with sodium methoxide affording (\pm) -griseofulvin (**1**).

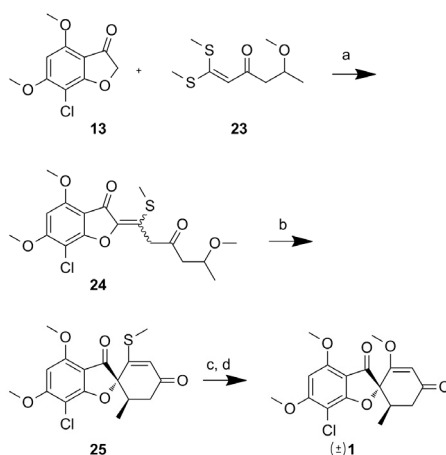
Pirrung et al. (1991)⁴¹ reported the first total synthesis of (+)-griseofulvin (**1**) (see Scheme 8). Reacting the triether **26**, with Mander's reagent followed by diazo transfer afforded **27**, which upon treatment with 5% rhodium pivalate catalyst yielded, through a sigmatropic rearrangement, the 3-coumarone **28**. Conversion to **29** followed by Dieckmann cyclisation and diazomethane treatment completed the total synthesis of (+)-griseofulvin (**1**).



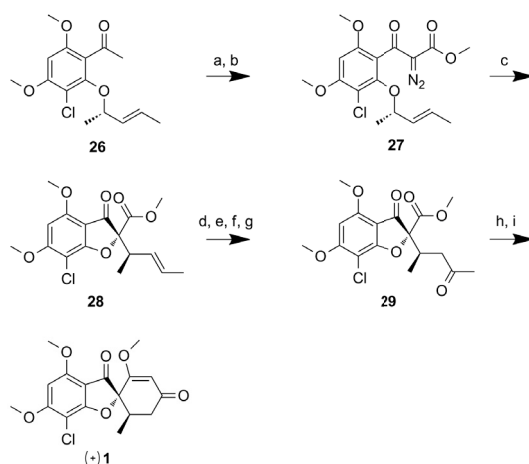
Scheme 5: (a) NaH, THF, HMPA; (b) thiophenol, *p*-toluenesulfonic acid, benzene; (c) *m*-CPBA, CH₂Cl₂; (d) toluene, 100-135 °C (e) H₂, 10% Pd/C, EtOH



Scheme 6: (a) HCl, TiCl₄, CH₂Cl₂



Scheme 7: (a) *t*-BuOK, DMF, 0 °C; (b) activated alumina, Et₂O (c) *m*-CPBA, CH₂Cl₂, 0 °C; (d) MeONa, MeOH, benzene, 0 °C



Scheme 8: (a) LHMDS, NCCO₂Me, THF, -78 °C; (b) MsN₃, Et₃N; (c) Rh₂(piv)₄, benzene; (d) O₃, CH₂Cl₂, MeOH, (CH₃)₂S; (e) Ph₃C(CH₃)CO₂-*t*-Bu, THF; (f) TFA; (g) diphenyl phosphorazidate, Et₃N; toluene, then HCl/H₂O; (h) NaOMe, MeOH; (i) CH₂N₂, THF

2.2 Total Syntheses of Griseofulvin Analogs

Ring A and B as well as position 6' of griseofulvin (**1**) are not easily modified from the natural product and therefore analogs of these types have been obtained by total synthesis. The syntheses have been completed using a number of the methods developed for the total synthesis of griseofulvin. The strategies used are oxidative cyclisation,^{42,43,44,45} Diels-Alder,^{38,46} double Michael addition,⁴⁷ Dieckmann cyclization,⁴⁸ and the improved Michael addition method.⁴⁹ The analogs synthesized by means of total synthesis can be seen in Figure 2.2 and Table 2.1.

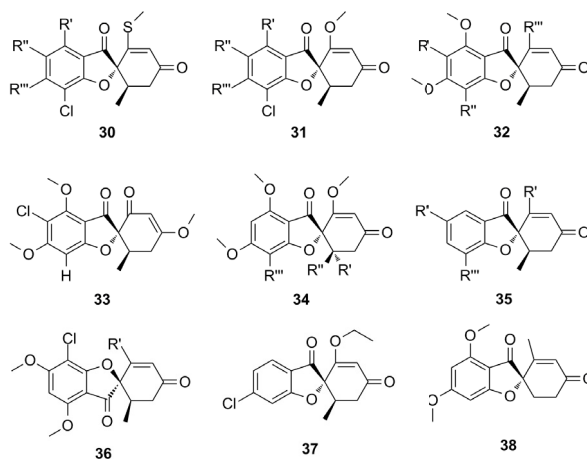


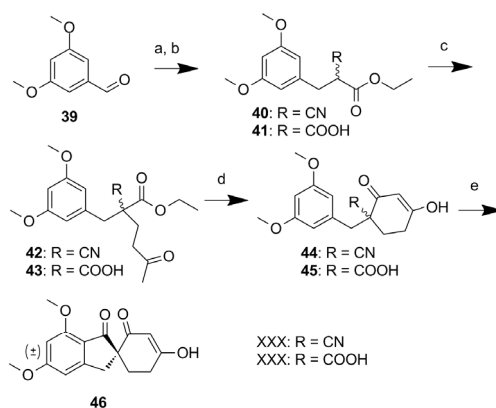
Figure 2.2: Racemic analogs synthesized through total synthesis. The structures are shown in Table 2.1.

Compound	R'	R''	R'''	Reference
30	OMe	H	H	49
30	H	OMe	H	49
30	H	H	OMe	49
30	OMe	OMe	OMe	49
30	OMe	Me	OMe	49
30	OMe	Cl	OMe	49
31	OMe	H	H	49
31	H	OMe	H	49
31	H	H	OMe	49
31	OMe	OMe	OMe	49
31	OMe	Me	OMe	49
31	OMe	Cl	OMe	49
31	OMe	COOEt	OMe	48
32	Cl	H	OMe	44,50,34,51,35
32	H	NO ₂	OMe	51
32	NO ₂	H	OMe	51

Continued on next page

Compound	R'	R''	R'''	Reference
32	H	Me	OMe	51
32	Cl	Cl	OMe	35
32	H	Cl	OMe	34
32	H	Br	OMe	34
32	H	F	OMe	34,44,45
32	H	Cl	OEt	47
32	Cl	Cl	Cl	35
33	-	-	-	51
34	Me	H	Cl	46,38,52
34	Et	H	Cl	46,52
34	H	Et	Cl	52
34	Me	Me	Cl	46
34	H	H	Cl	50,46,33,33
34	H	Pr	H	44
35	OMe	H	H	47
35	OEt	H	H	47
35	OMe	Cl	H	47
35	OEt	Cl	H	47
35	OMe	H	Cl	47
35	OEt	H	Cl	47
35	OEt	Cl	Me	47
36	OEt	-	-	47
36	O(CH ₂) ₂ Ome	-	-	47
37	-	-	-	47
38	-	-	-	42

Table 2.1: Racemic griseofulvin analogs acquired through total synthesis. See Figure 2.2 for structures.



Scheme 9: (a) R=CN: ethyl cyanoacetate, piperidine; R=COOH: diethyl malonate, piperidine, HOAc, benzene; (b) H₂, 10% Pd/C, EtOAc; (c) but-3-en-2-one, 1,2-dimethoxyethane, NaOMe; (d) NaOMe, MeOH; (e) ZnCl₂, HCl, ether.

Newman and Angier developed a novel strategy for the preparation of ring B analogs, with a Hoesch condensation as the crucial step (See Scheme 9).^{53,54,55,56} In the first step either a cyano or acid group was introduced, the former by condensation of **39** with ethyl cyanoacetate using catalytic amounts of piperidine, which after hydrogenation using 10% palladium on charcoal, afforded the nitrile **40**. The latter was synthesized by treatment of **39** with diethyl malonate, piperidine and acetic acid in benzene yielding the acid **41** after hydrogenation. Both **40** and **41** were then reacted further with the Michael acceptor but-3-en-2-one using sodium methoxide as base yielding **42** and **43**. A Dieckmann condensation formed the destined ring C (**44**) and **45** by treatment with sodium methoxide in methanol. The Hoesch condensation was performed by bubbling hydrogen chloride through a mixture of **44** or **45** and zinc chloride in ether, affording a position 2 carbon analog of griseofulvin (**46**). The position 1 carbon and sulphur analogs of griseofulvin can be seen in Figure 2.3 and Table 2.2.

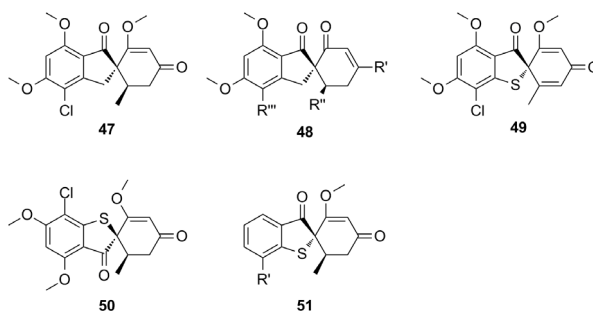


Figure 2.3: Ring B analogs synthesized through total synthesis. The compounds are shown in Table 2.2.

Compound	R'	R''	R'''	Reference
47	-	-	-	55
48	OH	H	H	53,54
48	OMe	Me	Cl	55
48	OH	Me	H	54
49	-	-	-	43
50	-	-	-	56
51	H	-	-	47
51	Cl	-	-	47
51	Me	-	-	47

Table 2.2: Ring B analogs prepared by total synthesis. See Figure 2.3 for structures.

2.3 Spectroscopic Studies of Griseofulvin and Analogs

Several articles do not contain synthesis of griseofulvin (**1**) or analogs thereof, but instead describe spectroscopic studies. Page and Staniforth thoroughly investigated the infrared absorption of a total of 188 griseofulvin analogs.^{57,58} The IR spectra were acquired in bromoform and very detailed absorption values are listed for all compounds. The authors were able to distinguish between analogs of the griseofulvin or isogriseofulvin form as well as between **1** and the position 2 epimer by IR.

A comprehensive study of the mass spectra of griseofulvin and analogs of griseofulvin have been carried out by Ballantine and Fenwick.⁵⁹ 60 MHz ¹H NMR was used in a study by Arison et al.⁶⁰ covering griseofulvin and 27 analogs, exploring the at the time, new technique while assigning groups based on differences between the analogs. Another NMR study by Green et al.⁶¹ including 43 analogs concluded that the assignment of the 4 and 6 methoxy groups by Arison et al. was faulty. The assignment by Green et al. was confirmed by Rønneest et al.⁶² by 2D NMR and single crystal X-ray analysis of the 4 and 6 phenols of **1**.

The conformation of griseofulvin and the 4'-alcohol analog of **1** in solution (CDCl₃) was determined by Levine and Hicks^{63,64} and they proceeded to investigate the ¹³C-NMR of griseofulvin and four analogs.⁶⁵ NMR has also been used in the study of the biosynthesis of griseofulvin using deuterium labeled analogs.⁶⁶ Hansch and Lien⁶⁷ used griseofulvin analogs in a larger modeling study of the LogP value of anti-fungal agents. These values were later used by Juvalle et al.⁶⁸ in a 2D-QSAR study using the curling of hyphae by the analogs reported by Crosse et al.⁶⁹ It was concluded by Crosse et al. that there were no correlation between the potency for curling of hyphae and the anti-fungal activity of analogs.

2.4 Tables of Griseofulvin Analogs

Numerous analogs of griseofulvin have been synthesized. In the following tables all occurrences in the literature, in either English or German, of griseofulvin and analogs are shown.

2.4.1 Griseofulvin Analogs with Modifications at the 2' Position

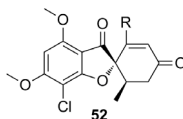


Figure 2.4: Griseofulvin analogs with modifications at the 2' position. See Table 2.3.

Structure	R	Reference
52	H	57,58,61,70,71,72,73
52	Me	70
52	OE _t	57,61,71,68,60,74,67,75
52	OP _r	74,70,71,57,68,76,75,67
52	O <i>i</i> P _r	57,75
52	OB _u	57,68,76,71,67,75
52	OPentyl	75
52	OH _{ex} yl	57,75
52	OAl _l	57,68,67
52	OCH ₂ - <i>cy</i> -propyl	57,75
52	O- <i>cy</i> -pentyl	57,75
52	OP _h	57,68,75,67
52	OB _n	57,68,75,67
52	O(4-Methyl-B _n)	75
52	O(CH ₂) ₂ B _n	75
52	Biphenylmethoxy	75
52	1-Adamantylmethoxy	75
52	OC ₆ H ₇ O ₅ Ac ₄ -A	71
52	B	58,61
52	NH ₂	77,71,76
52	NHMe	77,61
52	NHE _t	77
52	NC ₄ H ₈	77
52	Cl	57,58,61,78,75
52	(CH ₂) ₂ Br	79
52	(CH ₂) ₂ ¹²⁵ I	79
52	SMe	58,78,61,80
52	SE _t	78,80
52	SP _r	78,80

Continued on next page

Structure	R	Reference
52	SiPr	80
52	SBu	78
52	SAllyl	78
52	SPh	78
52	SC ₄ H ₉	80
52	SBn	78,75
52	S(CH ₂) ₂ OH	78
52	S(CH ₂) ₂ - <i>C</i>	78
52	S- <i>B</i>	78
52	S(CH ₂) ₂ NEt ₂	78

Table 2.3: Griseofulvin analogs with modifications at the 2' position. See Figure 2.4. *A* is glucose, *B* is pyrrolidin-1-yl and *C* is phthalimido.

2.4.2 Griseofulvin Analogs with Modifications at the 2' and 3' position

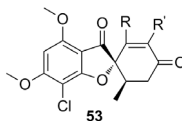


Figure 2.5: Griseofulvin analogs with modifications at the 2' and 3' position. See Table 2.4.

Structure	R	R'	Reference
53	OMe	Me	57,61,74,68,67
53	OMe	Pr	68,67
53	OMe	All	68,74,67
53	OMe	Bn	74,57,68,67
53	OMe	CH ₂ CH ₂ COCH ₃	74
53	OMe	NO ₂	81
53	OMe	NHBn	81
53	OMe	NHAc	81
53	OMe	Cl	57,60,61,68,67,82
53	OMe	Br	82,57,68,67
53	OMe	I	82,57,68,67,75
53	OEt	Bn	74,68,67
53	OEt	Et	74,57,68,67
53	OEt	Cl	82,68,67
53	OEt	Br	82,68,67
53	OEt	I	82,68,67
53	OPr	Me	74
53	OPr	Pr	74,68,67
53	OPr	Bn	74,68,67
53	OPr	Cl	82
53	OPr	Br	82,68,67
53	OPr	I	82,68,75,67
53	OBu	Bn	74,68,67
53	OBu	Cl	82
53	OBu	Br	82
53	OBu	I	82
53	O(CH ₂) ₃ Br	Bn	74
53	OBn	Br	82
53	OBn	Cl	82
53	OBn	I	82,75
53	Cl	Cl	57,78
53	Cl	Br	57,78
53	Cl	Bn	57,78

Continued on next page

Structure	R	R'	Reference
53	Cl	Pr	^{57,78}
53	SMe	Br	⁷⁸
53	SMe	Cl	⁷⁸
53	SEt	Cl	⁷⁸
53	SPr	Cl	⁷⁸
53	SBu	Cl	⁷⁸
53	SAll	Cl	⁷⁸
53	SBn	Cl	⁷⁸
53	SMe	Bn	⁷⁸
53	SEt	Bn	⁷⁸
53	SPr	Bn	⁷⁸
53	SBu	Bn	⁷⁸
53	SAll	Bn	⁷⁸
53	SBn	Bn	⁷⁸
53	SMe	Pr	⁷⁸
53	SPr	Pr	⁷⁸
53	SBu	Pr	⁷⁸
53	SAll	Pr	⁷⁸
53	SBn	Pr	⁷⁸

Table 2.4: Griseofulvin analogs with modifications at the 2' and 3' position. See Figure 2.5.

2.4.3 Griseofulvin Analogs with Modifications at the 5' Position

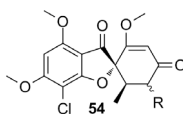


Figure 2.6: Griseofulvin analogs with modifications at the 5' position. See Table 2.5.

Structure	R	Reference
54	Br	83,84,85
54	Cl	83
54	I	83
54	F	83
54	CHO	83,86,84
54	OH	87
54	COCO ₂ Me	83
54	COCO ₂ Et	83
54	OCOMe	84
54	SO ₂ CH ₃	85
54	OMe	84
54	SMe	86
54	=CH ₂	86
54	CN	86
54	=NNHPh	86
54	CH=NNH(CH ₃) ₂	86
54	CH=NOH	86
54	NC ₄ H ₈	86
54	N ₂	84
54	SPh	86
54	SCOCH ₃	86,84
54	NC ₅ H ₁₀	86
54	Me	86

Table 2.5: Griseofulvin analogs with modifications at the 5' position. See Figure 2.6.

2.4.4 Griseofulvin Analogs with Modifications at the 4 Position

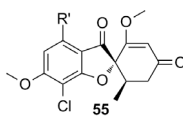


Figure 2.7: Griseofulvin analogs with modifications at the 4 position. See Table 2.6.

Structure	R	Reference
55	OH	75,62
55	Me	61
55	OE _t	88,61,57,75
55	OP _r	88
55	O <i>i</i> P _r	88,61
55	OB _u	88
55	OAc	61
55	All	68
55	CH ₂ Ph	68
55	A	61
55	OH	81
55	OAll	88,67
55	OB _n	88,75,67
55	O(CH ₂) ₂ NMe ₂	88
55	O(CH ₂) ₂ NEt ₂	88
55	OCH(Me)CH ₂ NMe ₂	88
55	OCH ₂ CONH- <i>p</i> -Cl-C ₆ H ₄	88
55	OC ₂ CONHCH ₂ Ph	88
55	OCH ₂ CH ₂ NC ₄ H ₈	88
55	OCH ₂ CH ₂ NC ₅ H ₁₀	88
55	OCH ₂ CH ₂ N ⁺ (Me) ₂ (Et)Br ⁻	88

Table 2.6: Griseofulvin analogs with modifications at the 4 position. See Figure 2.7. A is pyrrolidin-1-yl

2.4.5 Griseofulvin Analogs with Modifications at the 5 Position

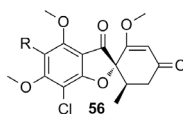


Figure 2.8: Griseofulvin analogs with modifications at the 5 position. See Table 2.7.

Structure	R	Reference
56	Cl	58,61,88
56	Br	57,58,89,82
56	NO ₂	58,90,81,89,75
56	NH ₂	81,90,75
56	Pr	57,61
56	NHBn	75

Table 2.7: Griseofulvin analogs with modifications at the 5 position. See Figure 2.8.

2.4.6 Griseofulvin Analogs with Modifications at the 6 Position

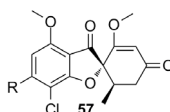


Figure 2.9: Griseofulvin analogs with modifications at the 6 position. See Table 2.8.

Structure	R	Reference
57	OE _t	57,68,88,67
57	OPr	68,88,67
57	O <i>i</i> Pr	61,88
57	OBu	68,88
57	OAc	61
57	OAl _l	68,88,67
57	OBn	88
57	OC ₁₆ H ₃₃	88
57	OCH ₂ COOCH ₃	88
57	A	61
57	OH	81,91,62

Table 2.8: Griseofulvin analogs with modifications at the 6 position. See Figure 2.9. A is pyrrolidin-1-yl

2.4.7 Griseofulvin Analogs with Modifications at Position 4 and 6

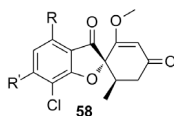


Figure 2.10: Griseofulvin analogs with modifications at positions 4 and 6. See Table 2.9.

Structure	R	R'	Reference
58	SMe	SMe	77,88
58	OEt	OEt	57,61

Table 2.9: Griseofulvin analogs with modifications at positions 4 and 6. See Figure 2.10.

2.4.8 Griseofulvin Analogs with Modifications at Position 5 and 7

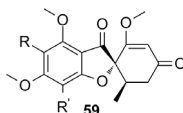


Figure 2.11: Griseofulvin analogues with modifications at ring A. See Table 2.10.

Structure	R	R'	Reference
59	H	F	60
59	NO ₂	H	81
59	All	H	57
59	NHBn	H	81
59	NH ₂	H	81
59	Cl	H	57,60,82
59	H	NH ₂	81
59	H	NO ₂	81

Table 2.10: Griseofulvin analogs with modifications at position 5 and 7. See Figure 2.11.

2.4.9 Isogriseofulvin Analogs with Modifications at the 4' Position

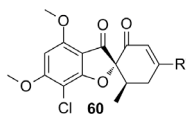


Figure 2.12: Isogriseofulvin analogs with modifications at the 4' position. See Table 2.11.

Structure	R	Reference
60	H	57,58,61
60	OMe	57,58,81,61,71
60	OEt	57,74,60,61,71
60	OPr	57,71,74
60	O <i>i</i> Pr	57,60
60	OBu	57,71
60	OHexyl	57,71
60	OAll	57
60	OPh	57
60	OBn	57
60	<i>A</i>	58
60	<i>B</i>	58
60	<i>C</i>	58,61
60	NH ₂	77,78
60	NHMe	61,77
60	NHBu	77
60	NEt ₂	77
60	NC ₄ H ₈	77
60	NC ₅ H ₁₀	77
60	NC ₄ H ₈ O	77
60	NMe(CH ₂) ₂ NEt ₂	77
60	NH(CH ₂) ₂ NC ₄ H ₈ O	77
60	SMe	58,80
60	SEt	78,80
60	SPr	80
60	S <i>i</i> Pr	80
60	SC ₄ H ₉	80
60	SPh	78
60	SBn	78
60	S(CH ₂) ₂ NEtCH ₃	78
60	O(CH ₂) ₂ Br	79
60	Cl	78,58,57,61

Table 2.11: Isogriseofulvin analogs with modifications at the 4' position. See Figure 2.12. *A* is phthalimido, *B* is 2-(2-imidazolyl), *C* is pyrrolidin-1-yl

2.4.10 Isogriseofulvin Analogs with Modifications at the 3' and 4' Positions

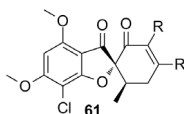


Figure 2.13: Isogriseofulvin analogs with modifications at the 3' and 4' position. See Table 2.12.

Structure	R	R'	Reference
61	Me	OMe	74,57,61
61	Me	OMe	74
61	Bn	OMe	74,57
61	(CH ₂) ₂ COCH ₃	OMe	74
61	Cl	OMe	78,61,82,57
61	Br	OMe	82,57,61
61	I	OMe	82,57
61	Et	OEt	74,57
61	Bn	OEt	74
61	Cl	OEt	82
61	Br	OEt	82
61	I	OEt	82
61	Me	OPr	74
61	Pr	OPr	74
61	Bn	OPr	74
61	Cl	OPr	82
61	Br	OPr	82
61	I	OPr	82
61	Bn	OBu	74
61	Cl	OBu	82
61	Br	OBu	82
61	I	OBu	82
61	Bn	OAll	74
61	Cl	OAll	82
61	Br	OAll	82
61	I	OAll	82
61	Cl	OBn	82
61	Br	OBn	82
61	I	OBn	82
61	Bn	O(CH ₂) ₃ Br	74
61	Cl	NH ₂	78
61	Br	NH ₂	78
61	Bn	NH ₂	78
61	Pr	NH ₂	78

Continued on next page

Structure	R	R'	Reference
61	Cl	Cl	⁵⁷
61	Br	Cl	^{78,57}
61	Bn	Cl	⁵⁷

Table 2.12: Isogriseofulvin analogs with modifications at the 3' and 4' position. See Figure 2.13.

2.4.11 Isogriseofulvin Analogs with Modifications at the 4, 5 and 6 Positions

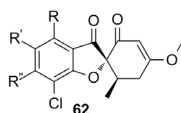


Figure 2.14: Isogriseofulvin analogs with modifications at the 4, 5 and 6 positions. See Table 2.13.

Structure	R	R'	R''	Reference
62	OMe	H	OEt	⁵⁷
62	OEt	H	OEt	⁵⁷
62	OEt	Cl	OMe	⁸⁸
62	OPr	Cl	OMe	⁸⁸
62	O <i>i</i> Pr	Cl	OMe	⁸⁸
62	OBu	Cl	OMe	⁸⁸
62	OAll	Cl	OMe	⁸⁸
62	OBn	Cl	OMe	⁸⁸

Table 2.13: Isogriseofulvin analogs with modifications at the 4, 5 and 6 positions. See Figure 2.14.

2.4.12 Analogs of both Griseofulvin and Isogriseofulvin with Modifications at Ring A and C

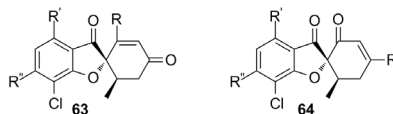


Figure 2.15: Analogs of both griseofulvin and isogriseofulvin with modifications at ring A and C. See Table 2.14.

Structure	R	R'	R''	Reference
63	OEt	OMe	OEt	71
63	NC ₄ H ₈	NC ₄ H ₈	OMe	77
63	NC ₄ H ₈	OMe	NC ₄ H ₈	77
63	OEt	OMe	OEt	57
63	OEt	OEt	OEt	57
64	OEt	OMe	OEt	57
64	OEt	OEt	OEt	57
64	OMe	NC ₄ H ₈	OMe	77

Table 2.14: Analogs of both griseofulvin and isogriseofulvin with modifications at ring A and C. See Figure 2.15.

2.4.13 Griseofulvic Acid with Modifications at the 3' Position

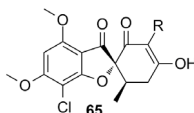


Figure 2.16: Analogs of griseofulvic acid with changes at the 3' position. See Table 2.15.

Structure	R	Reference
65	Me	58,74,89
65	Et	58
65	Pr	58
65	OPr	89
65	(CH ₂) ₂ Ac	58,74
65	NO ₂	58,81,89
65	NH ₂	81
65	NHAc	58
65	NHBn	58,81
65	Bn	58,74

Continued on next page

Structure	R	Reference
65	Cl	58,82
65	Br	82,58,78
65	I	58,82,89
65	A	61

Table 2.15: Analogs of griseofulvic acid with modifications at the 3' position. See Figure 2.16. *A* is the sodium salt of the enol form.

2.4.14 Disubstituted Griseofulvic Acid at the 3' Position

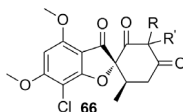


Figure 2.17: Analogs of griseofulvic acid with changes at the 3' position. See Table 2.16.

Structure	R	R'	Reference
66	Cl	Cl	82
66	Me	Me	74,58,61,75
66	Bn	Bn	74,58

Table 2.16: Analogs of griseofulvic acid with modifications at the 3' position. See Figure 2.17

2.4.15 Griseofulvic Acid with Modifications at the 5, 7 and 3' Positions

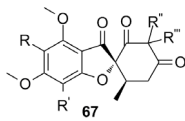


Figure 2.18: Analogs of griseofulvic acid with changes at the 5, 7 and 3' positions. See Table 2.17.

Structure	R	R'	R''	R'''	Reference
67	H	H	NH ₂	H	81
67	H	H	NHBn	H	58,81
67	Cl	Cl	Cl	H	58,82
67	Br	Cl	Br	H	58,82
67	Cl	Cl	H	H	82
67	Br	Cl	H	H	82
67	Cl	Cl	Cl	Cl	58,82
67	Br	Cl	Br	Br	58,82

Table 2.17: Analogs of griseofulvic acid with modifications at the 5, 7 and the 3' position. See Figure 2.18.

2.4.16 Griseofulvic Acid with Modifications at the 6 position

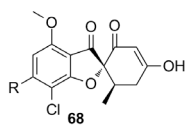


Figure 2.19: Analogs of griseofulvic acid with modifications at the 6 position. See Table 2.18.

Structure	R	Reference
68	OH	58
68	OE _t	58
68	OP _r	58,88
68	O <i>i</i> P _r	58,88
68	OB _u	58,88
68	OC ₁₆ H ₃₃	58,88
68	OAl _l	58,88
68	OB _n	58,88
68	A	58
68	OCH ₂ COOH	88

Continued on next page

Structure	R	Reference
68	$(\text{CH}_2)_2\text{NEt}_2$	⁸⁸

Table 2.18: Analogs of griseofulvic acid with modifications at the 6 position. See Figure 2.19. *A* is pyrrolidin-1-yl

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CHAPTER 3

Synthesis and Structure-Activity Relationship of Griseofulvin Analogues as Inhibitors of Centrosomal Clustering in Cancer Cells

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The supporting information from Chapter 3 can be seen in Appendix C

Synthesis and Structure–Activity Relationship of Griseofulvin Analogues as Inhibitors of Centrosomal Clustering in Cancer Cells

Mads H. Rønne^{†,‡}, Blanka Rebacz,[§] Lene Markworth,[†] Anette H. Terp,^{‡,§} Thomas O. Larsen,[‡] Alwin Krämer,[§] and Mads H. Clausen^{*,†}

Department of Chemistry, Technical University of Denmark, Kemitorvet, Building 201, DK-2800 Kgs. Lyngby, Denmark, Center for Microbial Biotechnology, Department of Systems Biology, Søtofts Plads, Building 221, DK-2800 Kgs. Lyngby, Denmark, Clinical Cooperation Unit for Molecular Hematology/Oncology, German Cancer Research Center (DKFZ) and Department of Internal Medicine V, University of Heidelberg, Im Neuenheimer Feld 280 (TP4) 69120 Heidelberg, Germany

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Griseofulvin was identified as an inhibitor of centrosomal clustering in a recently developed assay. Centrosomal clustering is an important cellular event that enables bipolar mitosis for cancer cell lines harboring supernumerary centrosomes. We report herein the synthesis and SAR of 34 griseofulvin analogues as inhibitors of centrosomal clustering. The variations in the griseofulvin structure cover five positions, namely the 4, 5, 2', 3', and 4' positions. Modification of the 4 and 5 positions affords inactive molecules. The enol ether must be at the 2' position, and the 4' position needs to be sp² hybridized. The most active analogues were the 2'-benzyloxy and 2'-(4-methylbenzyloxy) analogues as well as the oxime of the former with a 25-fold increase of activity compared to griseofulvin. Comparison of the results obtained in this work with prior reported growth inhibition data for dermatophytic fungi showed both similarities and differences.

Introduction

Griseofulvin (**1**, Figure 1) is a classic antifungal agent^{1,2} used clinically for the treatment of dermatomycoses. Since the isolation of griseofulvin in 1939,³ it has attracted a lot of attention and more than a hundred papers describing analogue synthesis as well as structure–activity relationship (SAR⁴) studies relating to antifungal activity have been published. Since 1950, more than 400 analogues have been disclosed covering most positions and many have displayed significantly increased activity in published antifungal assays. More recently, griseofulvin has been the object of increased interest due to its activity toward a range of mammalian cancer cell lines.⁴ Griseofulvin has been shown to be cytotoxic by itself but also to potentiate the activity of another anticancer drug, nocodazole.^{5,6} The cytotoxicity of three 2' analogues in Chinese hamster V79 cells has previously been reported by Oda.⁷

We have recently developed an assay⁸ to identify small molecules that inhibit centrosomal clustering, a mechanism necessary for successful bipolar mitoses in human cancer cell lines with supernumerary centrosomes.^{9,10} Healthy mammalian cells harbor two centrosomes during mitosis that function as mitotic spindle poles to ensure accurate chromosome segregation, whereas the majority of human malignancies contain multiple centrosomes. Supernumerary centrosomes can form multipolar spindles leading to aberrant mitoses with consecutive chromosome missegregation, eventually resulting in apoptosis. In most human cancer cell lines, this spindle multipolarity is overcome through centrosomal clustering, giving rise to two

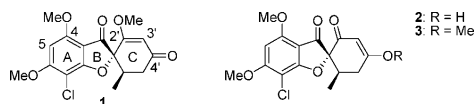


Figure 1. Structures of griseofulvin **1**, griseofulvic acid **2**, and isogriseofulvin **3**.

functional spindle poles and thereby allowing for successful mitosis.¹¹ As this phenotype is specific to cancer cells, there is a possibility that the compounds identified in our assay will target cancer cells selectively without interfering with healthy cells.

Microtubules are vital components of centrosomes, and compounds interacting with the dynamics of tubulin polymerization and depolymerization are a major category of anticancer agents including vinca alkaloids and taxanes.¹² Griseofulvin is known to interact with tubulin,¹³ but the mode of action for this inhibition is not fully understood although a few theories have been proposed.^{4,5,14}

We have tested 34 griseofulvin analogues in our assay, of which nine are novel, affording the first SAR study of this compound class with regard to anticancer activity. The analogues vary at five positions of the griseofulvin structure, namely the 4, 5, 2', 3', and 4' positions. All analogues described in this paper have been prepared from commercially available griseofulvin in one to four synthetic steps.

Chemistry

The phenol **4** was synthesized by treatment of **1** with MgI₂ in diethyl ether and toluene.¹⁵ Alkylation of **4** with either ethyl or benzyl bromide using NaH as base afforded elongated analogues **5** and **6** (Scheme 1). Nitration¹⁶ of **1** gave **7**, which could be reduced to the amine **8** with iron powder.¹⁷ Reductive amination with benzaldehyde yielded analogue **9** (Scheme 1).

Two synthetic routes have been utilized for the preparation of the 2' enol ether analogues of **1**. Direct camphorsulfonic acid (CSA) mediated solvolysis of griseofulvic acid (**2**)¹⁸ afforded

* To whom correspondence should be addressed. Phone: +45 45252131. Fax: +45 45933968. E-mail: mhcl@kemi.dtu.dk.

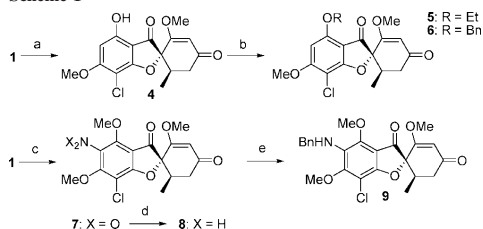
[†] Department of Chemistry, Technical University of Denmark.

[‡] Center for Microbial Biotechnology, Department of Systems Biology.

[§] Clinical Cooperation Unit for Molecular Hematology/Oncology, German Cancer Research Center (DKFZ) and Department of Internal Medicine V, University of Heidelberg.

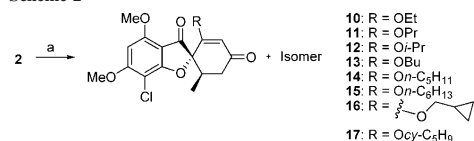
^{||} Abbreviations: CSA, camphorsulfonic acid; DBU, diaza(1,3)bicyclo[5.4.0]undecane; NIS, *N*-iodosuccinimide; NOE, nuclear Overhauser effect; SAR, structure–activity relationship; TESOTf, triethylsilyl trifluoromethanesulfonate.

Scheme 1^a



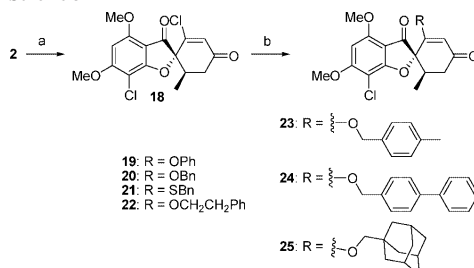
^a (a) MgI_2 , toluene, Et_3O ; (b) RBr , tetrabutylammonium iodide, NaH , DMF ; (c) HNO_3 , Ac_2O ; (d) Fe , HOAc ; (e) PhCHO , NaBH(OAc)_3 , CH_2Cl_2 .

Scheme 2^a



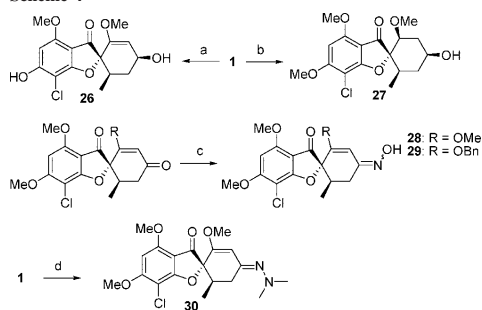
^a (a) ROH , CSA , dioxane (**16** and **17**).

Scheme 3^a



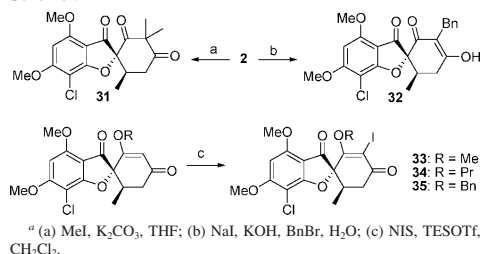
^a (a) LiCl , POCl_3 , dioxane; (b) ROH , DBU , THF , or dioxane.

Scheme 4^a



^a (a) NaBH_4 , CeCl_3 , MeOH ; (b) H_2 , Pt/C , EtOAc ; (c) hydroxylamine, NaOAc , EtOH , DMSO ; (d) Me_2NNH_2 , HOAc , toluene.

Scheme 5^a



^a (a) MeI , K_2CO_3 , THF ; (b) NaI , KOH , BnBr , H_2O ; (c) NIS , TESOTf , CH_2Cl_2 .

compounds **10–15** plus their 4' enol ether isomers. Dioxane was used as cosolvent for alcohols with higher boiling points, leading to **16** and **17**. This method was generally applicable to the synthesis of analogues with straight chain vinyl ethers in yields of 14–22% (Scheme 2).

Bulkier alcohols like benzyl alcohol and cyclopentanol led to unsatisfactory yields of 2–4%. An attractive alternative was the known¹⁹ route through the 2' vinyl chloride **18**, which relies on a base mediated addition of the alcohol followed by elimination of chloride (Scheme 3). Vinyl chloride **18** is accessible by treatment of **2** with lithium chloride in phosphoryl chloride. We found using dioxane as cosolvent convenient because this allowed us to reduce the amount of phosphoryl chloride to 5 equiv, greatly facilitating the workup procedure and the scale-up to multigram amounts.

The addition of alcohols to **18** performed well in DMF , THF , and dioxane with comparable yields and THF or dioxane was used as solvent for all subsequent experiments. NaH and Cs_2CO_3 were tested as bases, but in the end, diaza(1,3)bicyclo[5.4.0]undecane (DBU) turned out to be the most efficient base and it was used for preparation of analogues **19–25** in yields of 2–93% (Scheme 3).

The 4' alcohol **26** was derived from **1** using a slight modification of a known method²⁰ (Scheme 4). Analogue **27**

was available by reduction of **1** with hydrogen catalyzed by platinum on charcoal, an improvement of a published procedure.²¹ The oximes **28** and **29** were derived from the parent ketones by treatment with hydroxylamine hydrochloride in ethanol and DMSO , a slight modification of the method of Delgado et al.¹⁷ and isolated as inseparable 1:1 mixtures of geometrical isomers. In a similar fashion, the hydrazine **30** was synthesized by heating **1** with N,N -dimethyl hydrazine and acetic acid in toluene. The major product was the *E* analogue **30** with the geometry distinguished by NOE in proton NMR. The $\text{H}^{3'}$ proton of the two isomers showed two distinct signals in NMR. No dipolar coupling between a hydrazine methyl group and $\text{H}^{3'}$ could be observed for the pure analogue **30**, but a strong NOE was detected for the isomer.

3',3'-Dimethyl griseofulvic acid (**31**) was derived from **2** treated with K_2CO_3 and MeI ²² (Scheme 5). Analogue **32** was synthesized from **2** by reaction with benzyl bromide, NaI , and KOH .²² Treatment of **1** with N -iodosuccinimide (NIS) and triethylsilyl triflate (TESOTf) afforded **33**, and the 3'-iodo analogues **34** and **35** were obtained using the same convenient conditions. This type of compounds have previously been prepared by treating **2** with iodine monochloride and 2 equiv of KOAc ,²³ followed by reacting the intermediate with an excess of an appropriate diazo alkane.²⁴

Results and Discussion

Compounds **1–35** were evaluated for their ability to inhibit centrosomal clustering in our cellular assay⁸ (Table 1). The percentages of cells with multipolar spindles were measured in triplicate at 14 different concentrations (100 μM to 12 nM), and IC_{50} values were calculated with an IC_{50} of 24 μM for griseofulvin (**1**). Analogues with IC_{50} values higher than 100 μM are regarded as inactive.

Table 1. Compounds Included in the Article and Their IC₅₀ Values within a 95% Confidence Interval

Analogue	IC ₅₀ (μ M)	Analogue	IC ₅₀ (μ M)	Analogue	IC ₅₀ (μ M)	Analogue	IC ₅₀ (μ M)
1	24 \pm 2	10	6.6 \pm 0.5	19	1.5 \pm 0.1	28	17 \pm 1
2	>100	11	6.6 \pm 0.5	20	0.9 \pm 0.1	29	0.9 \pm 0.1
3	>100	12	6.8 \pm 0.5	21	1.3 \pm 0.1	30	27 \pm 2
4	>100	13	2.5 \pm 0.2	22	15 \pm 2	31	>100
5	>100	14	2.8 \pm 0.2	23	0.9 \pm 0.1	32	>100
6	>100	15	4.0 \pm 0.2	24	2.9 \pm 0.2	33	>100
7	>100	16	1.8 \pm 0.1	25	3.7 \pm 0.2	34	2.0 \pm 0.1
8	>100	17	1.3 \pm 0.1	26	>100	35	>100
9	>100	18	-	27	>100		

At ring A, the 4 and 5 positions were altered. Elongation at the 4 position resulted in no activity for either the ethyl (**5**) or benzyl (**6**) ethers. Introduction of a nitro (**7**), amine (**8**), or benzyl amine (**9**) group at the 5 position negates all activity, indicating that bulkier groups at these two positions are not tolerated. Compound **6** has previously been tested for growth inhibition against dermatophytes (*Epidermophyton floccosum*, *Trichophyton interdigitale*, *T. persicolor*, *T. mentagrophytes*, *T. rubrum*, and *Microsporum canis*), and **7** and **8** have been tested against the latter three in addition to *T. terrestre* and all three were found to be less active than **1**.^{17,25}

The 2', 3', and 4' positions of the C ring have been altered in this study. Solvolysis of **2** with a given alcohol affords two isomers with the enol ether positioned at either the 2' or 4' position. We have tested altogether 11 analogues with a 4' enol ether as in isogriseofulvin (**3**), including the isomers of **10–17** and **20**, and all have been inactive (data not shown). This strongly indicates that active analogues should have the enol ether at the 2' position. The activity of the 4' enol ether isomers of **3**, **10**, **13**, and **19** toward a range of dermatophytes (*Trichophyton mentagrophytes*, *T. interdigitale*, *T. rubrum*, *T. persicolor*, *Microsporum canis* and *Epidermophyton floccosum*) have been published and all showed lower activity than **1**.²⁵

Several analogues with modifications at the 2' position have been prepared, and elongation of the methoxy group in **1** (analogues **10–15**) increases the activity, with a maximum around 4 carbon atoms.

The activity can be further increased by the introduction of bulkier groups. The cyclopropylmethoxy (**16**) and cyclopentoxo (**17**) analogues have IC₅₀ values of 1.8 and 1.3 μ M, comparable to the phenoxy (**19**) and benzylthio (**21**) analogues with 1.5 and 1.3 μ M. The most active 2' analogues are the benzyloxy (**20**) and 4-methylbenzyloxy (**23**) compounds, both with an IC₅₀ value of 0.9 μ M. While there is little difference between **19** and **20**, the phenylethoxy analogue **22** is significantly less potent with an IC₅₀ value of 15 μ M. The introduction of even bulkier groups such as 4-biphenylmethoxy (**24**) and adamantylmethoxy (**25**) affords IC₅₀ values of 2.9 and 3.7 μ M, higher than the most active compounds but significantly more potent than **1**. Because our assay is whole-cell based, transport over the cell membrane potentially also influences the observed activity. The higher lipophilicity of **24** and **25** could render this transport easier, which would contribute to the observed increase in activity.

Growth inhibition data for the dermatophytes *Trichophyton mentagrophytes*, *T. interdigitale*, *T. rubrum*, *T. persicolor*, *Microsporum canis*, and *Epidermophyton floccosum* has been published for compounds **10**, **11**, **13**, **15**, **19**, and **20**.²⁵ The activities for **10** and **11** were equal to or lower than **1**, while **13** was more active against two strains (*T. interdigitale*, *T. persicolor*) and similar or less toward the remaining strains. Compound **15** showed increased activity against a single strain (*T. interdigitale*), and **19** and **20** were inactive.

A different phenotype was seen for **18**. Usually, cells are attached to the dish by focal adhesions. Treatment with **18** led to a detachment of the cell monolayer from the dish, but, interestingly, the cell–cell contacts were not affected. Because of this phenotype, induction of multipolar spindle formation could not be analyzed.

The 4' alcohol, griseofulvol (**26**), has no activity and neither has the 2'-methoxy 4'-alcohol analogue (**27**). The 4' position of **1** was altered to an oxime (**28**), giving rise to an increase in activity with an IC₅₀ of 17 μ M. To check for a possible synergistic effect, the 2' benzyloxy analogue **20** was also fitted with an oxime affording **29**, but the activity did not exceed that of **20** as the IC₅₀ value remained at 0.9 μ M. Introduction of a hydrazine group at the 4' position (**30**) led to a slight decrease in activity with an IC₅₀ of 27 μ M. No activity was reported for **26** toward dermatophytes *Trichophyton mentagrophytes* or *T. rubrum*, but for **28**, the activity toward those two strains and *T. terrestre* and *Microsporum canis* was comparable to griseofulvin.¹⁷

The results from the 4' analogues indicate that this position is important for the activity, as removal of the ketone renders the compounds (**26** and **27**) inactive. Taken together with the increase in activity for **28** and **29**, it suggests that the 4' position should be sp² hybridized.

Neither griseofulvic acid **2**, the 3',3'-dimethyl analogue **31**, nor 3'-benzyl griseofulvic acid (**32**) showed activity in our assay. Introduction of iodine at the 3' position had equivocal effects. 3'-Iodo griseofulvin (**33**) and 3'-iodo-2'-benzyloxy analogue **35** were inactive. For the 2'-propoxy-3'-iodo analogue **34**, the IC₅₀ found was 2.0 μ M, only a factor two higher than the most active compounds and lower than the 2'-propoxy analogue **11** itself. In antifungal screens, **33** shows lower activity toward *Trichophyton mentagrophytes*, *T. interdigitale*, *T. rubrum*, *T. persicolor*, *Microsporum canis*, and *Epidermophyton floccosum*,

where **34** has a lower activity against all save *T. interdigitale*, where the activity is the same as **1**.²⁵

Conclusion

We report here for the first time a collection of griseofulvin analogues tested for inhibition of centrosomal clustering in cancer cells. Of the 34 analogues tested, 13 were inactive, two had decreased activity, and 18 displayed increased activity as compared to griseofulvin, while one analogue showed a different phenotype characterized by detachment of the cell monolayer from the dish.

All modifications of the 4 and 5 positions resulted in no activity, and thus these positions should not be altered. It appears as if the 4' position needs to be sp² hybridized as reduction of the ketone results in no activity, whereas introduction of either an oxime (**28** and **29**) or a hydrazine (**30**) group leads to more active analogues. A total of 11 4' enol ether analogues have been tested, and all were inactive. The two inactive analogues **31** and **32** also indicate that modifications must preserve the conjugated system of **1** to be beneficial.

The 2' position has been explored the most, and elongation at this position increases the activity while introducing bulkier groups do so to an even larger extent. The optimal substituent seems to be a benzyloxy group (**20**) with the possibility of substitution on the aromatic ring. The phenylethoxy analogue **22** is 15 times less active than **20**, and a longer tether is thus not a viable option for increased activity.

The three iodo substituted analogues do not give a clear picture of the effect of substitution at the 3' position as the activity varies. We speculate that the iodine changes the conformation of the 2' substituent and that the benzyloxy group is shifted to a less desirable position, whereas the propyloxy group is moved to a more favorable position.

While comparing the SAR data obtained in this work for inhibition of centrosomal clustering with the growth inhibitory effect against seven dermatophytic fungi, some similarities are seen but also notable differences. Modification of the 4 and 5 positions in the A ring renders the analogues inactive with regard to both activities. For the C ring modifications, the 4' enol ether analogues displayed reduced or no activity in both types of assay. When modifying the 2' position, the activity toward dermatophytes is retained for the ethyl and propyl analogues. The butyl and hexyl analogues show increased activity toward some fungal strains and lower toward others. The inhibition of centrosomal clustering is increased for all straight chained 2' analogues, compared to griseofulvin. With bulkier substituents (**19** and **20**), lower activity is seen toward all dermatophyte strains, while the inhibition of centrosomal clustering is enhanced further.

Reduction of the 4' ketone makes the analogue inactive both toward dermatophytes and cancer. An oxime on the 4' position retains activity toward dermatophytes but increases the centrosomal clustering inhibition. Compound **33** with substitution in the 3' position is less active in our assay as well as against dermatophytes, while **34** has diminished antifungal activity but is 12 times more potent than **1** in our assay.

The results described herein demonstrate that the activity of griseofulvin as an inhibitor of centrosomal clustering can be enhanced by structural modifications, which renders this compound class interesting as potential anticancer agents. Furthermore, we have demonstrated that previously published data on

the antifungal activity of griseofulvin analogues does not correlate directly to their activity in mammalian cells.

Experimental Section

Starting materials, reagents, and solvents were purchased from Sigma-Aldrich Chemical Co. and used without further purification. Reactions involving air or moisture sensitive reagents were carried out under N₂, and flasks were dried by flame heating under reduced pressure. DMF, CH₂Cl₂, dioxane, and toluene were dried over 3 Å molecular sieves. Evaporation of solvents was done under reduced pressure (in vacuo). Purity of all compounds was found to be greater than 95% by LC-DAD-MS (see below).

NMR spectra were recorded using either a Varian Unity Inova 500 MHz spectrometer or a Varian Mercury 300 MHz spectrometer. ¹³C NMR spectra were recorded using either a Bruker AC 200 MHz or a Varian Mercury 300 MHz. Chemical shifts were measured in ppm and coupling constants in Hz, the field is indicated in each case. When CDCl₃ was used as solvent, the residual peak was used as internal reference at δ 7.27 for ¹H NMR and δ 77.00 for ¹³C NMR spectra. When DMSO-*d*₆ was used, the values were δ 2.50 for ¹H NMR and δ 39.43 for ¹³C NMR spectra.

IR spectra were recorded using a Perkin-Elmer 1600 series FTIR. All melting points are uncorrected. TLC was performed on aluminum sheets precoated with silica gel 60 F254 (Merck 1.05554.0001). Compounds were visualized by charring after dipping in a solution of 1% KMnO₄, 6.7% K₂CO₃, and 0.08% NaOH in water. UV visualization was done using a model UVGL-25 Mineralight lamp.

EIMS were recorded by direct inlet to a GCMS-QP5000 gas chromatograph mass spectrometer from Shimadzu. High-resolution LC-DAD-MS was performed on an Agilent 1100 system equipped with a photodiode array detector (DAD) and coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass) with a Z-spray electrospray ionization (ESI) source and a LockSpray probe (M + H 556.2771) and controlled by MassLynx 4.0 software. LC-MS calibration from *m/z* 100–900 was done with a PEG mixture. Standard separation involved a LUNA 2 column with an acetonitrile (50 ppm TFA) in water gradient starting from 15% to 100% over 25 min with a flow rate of 0.3 mL/min. Microanalyses were obtained from H. Kolbe, Mikroanalytisches Laboratorium, Mülheim/Ruhr, Germany.

For the determination of IC₅₀ values, we tested the compounds in our assay⁸ for centrosomal clustering based on SSC114 cells, an oral squamous cell carcinoma cell line characteristic for this phenotype. Despite the presence of supernumerary centrosomes in 64.5% of SCC114 cells, only 3.6% of the cells in mitosis harbor multipolar spindles. The rest of the cell population cluster their centrosomes and undergo bipolar mitoses, making this cell line an ideal model system. SCC114 cells were grown in 96-well plates to near confluence and treated for 24 h with different griseofulvin analogues. The compounds were tested in triplicate at 14 different concentrations (100, 50, 25, 12.5, 6.3, 3.1, 2, 1, 0.4, 0.2, 0.1, 0.05, 0.02, and 0.01 μM). Cells were then fixed and examined by fluorescence microscopy. Then 100 mitotic cells per well were analyzed, with the percentage of mitotic cells with multipolar spindles being the read-out. The relationship between readout and the dose was described by a logistic model (eq 1):

$$p = (1 + \exp(-\alpha - \beta x))^{-1} \quad (1)$$

where *p* is the percentage of mitotic cells with multipolar spindles and *x* is the dose scaled according to $x = \ln(\text{dose} + 1)$. The dose axis was scaled to spread out the low concentration data. The IC₅₀ values and the corresponding 95% confidence intervals were determined from the fitted dose–response curves. The calculations were performed in Matlab 7.0.4.365 (The Mathworks) using the *glmfit* and *glmval* functions.

(2*S*,6*R*)-(7-Chloro-4,6-dimethoxy-5-benzylamino-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-ene-4'-one) (**9**). To a solution of 5-amino griseofulvin **8** (120 mg, 0.327 mmol) and

benzaldehyde (0.07 mL, 0.654 mmol) in CH_2Cl_2 (4 mL) was added a solution of sodium triacetoxymethylborohydride (104 mg, 0.490 mmol) in CH_2Cl_2 (4 mL) at 0 °C under nitrogen atmosphere. Acetic acid (90% aq, 0.03 mL, 0.654 mmol) was added and the mixture was stirred at 20 °C for 48 h. The mixture was washed with sat. aq. NaHCO_3 (15 mL) and water (15 mL). The combined aqueous phases were extracted with CH_2Cl_2 (3 \times 30 mL). The combined organic phases were dried (MgSO_4) and concentrated. The residue was purified by column chromatography (toluene: CH_2Cl_2 :EtOAc 30:30:1) to afford the product **9**. Yield: 26 mg (17%) (yellow needles); R_f (EtOAc:heptane 5:2): 0.54; mp: 79–80 °C. IR (KBr, cm^{-1}) 1709, 1664. ^1H NMR (CDCl_3) δ 7.31–7.18 (5H, m), 5.52 (1H, s), 4.30 (2H, s), 3.90 (3H, s), 3.86 (3H, s), 3.60 (3H, s), 2.93 (1H, dd, J = 16.1, 13.3 Hz), 2.85–2.75 (1H, m), 2.40 (1H, dd, J = 16.1, 4.1 Hz), 0.89 (3H, d, J = 6.5 Hz). ^{13}C NMR (CDCl_3) δ 196.8, 194.1, 171.1, 163.9, 156.8, 146.6, 140.2, 128.8 (2C), 128.2 (2C), 127.6, 110.2, 106.2, 105.2, 105.1, 90.1, 62.6, 60.5, 57.0, 51.5, 40.2, 36.8, 14.5. HRMS (ESI^+) calcd for $[\text{C}_{24}\text{H}_{22}\text{ClNO}_6]^+$ 458.1370, found 458.1371.

General Procedure for the Synthesis of Enol Ethers by Solvolysis (16 and 17). CSA (0.1 mmol, 0.1 equiv) was added to a solution of griseofulvic acid (0.6 mmol, 1 equiv), the appropriate alcohol (3 mmol, 5 equiv), and 1,4-dioxane (3 mL). The mixture was stirred at 100 °C for 6 h and then cooled to 20 °C. EtOAc (20 mL) was added to the solution, and the mixture was washed with sat. NaH_2PO_4 (20 mL) and then water (20 mL). The combined aqueous phases were extracted with EtOAc (3 \times 20 mL), dried (MgSO_4), and then concentrated. The residue was purified by column chromatography (toluene: CH_2Cl_2 :EtOAc 7:7:1) to afford the desired product and the isomer. When possible the product was recrystallized from EtOAc/Heptane.

(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(2'-cyclopropylmethoxy-6'-methyl-cyclohex-2'-ene-4'-one) (16). Yield: 86 mg (4%) (white crystals); R_f (EtOAc:heptane 5:1): 0.51; mp: 190–191 °C. IR (KBr, cm^{-1}) 1704, 1659, 1608. ^1H NMR (500 MHz, CDCl_3) δ 6.13 (1H, s), 5.47 (1H, s), 4.03 (3H, s), 3.98 (3H, s), 3.65 (2H, d, J = 6.5 Hz), 3.03 (1H, dd, J = 16.7, 13.5 Hz), 2.83 (1H, ddq, J = 13.5, 4.7, 6.6 Hz), 2.41 (1H, dd, J = 16.7, 4.7 Hz), 1.05–0.98 (1H, m), 0.96 (3H, d, J = 6.6 Hz), 0.50–0.43 (2H, m), 0.22–0.13 (2H, m). ^{13}C NMR (50 MHz, CDCl_3) δ 197.0, 192.5, 169.9, 169.6, 164.4, 157.6, 105.0 (2C), 97.1, 90.8, 89.3, 73.2, 56.9, 56.3, 39.9, 36.2, 14.2, 9.0, 2.7 (2C). HRMS (ESI^+) calcd for $[\text{C}_{20}\text{H}_{22}\text{ClO}_6]^+$ 393.1105, found 393.1108. Anal. ($\text{C}_{20}\text{H}_{21}\text{ClO}_6$): C, H.

(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(2'-cyclopentoxo-6'-methyl-cyclohex-2'-ene-4'-one) (17). Yield: 50 mg (4%) (yellow oil); R_f (EtOAc:heptane 5:1): 0.50. IR (KBr, cm^{-1}) 1705, 1652, 1615. ^1H NMR (500 MHz, CDCl_3) δ 6.11 (1H, s), 5.49 (1H, s), 4.56–4.51 (1H, m), 4.03 (3H, s), 3.97 (3H, s), 3.03 (1H, dd, J = 16.7, 13.5 Hz), 2.82 (1H, ddq, J = 13.5, 4.8, 6.7 Hz), 2.40 (1H, dd, J = 16.7, 4.8 Hz), 1.79–1.72 (2H, m), 1.72–1.64 (2H, m), 1.58–1.44 (4H, m), 0.95 (3H, d, J = 6.7 Hz). ^{13}C NMR (50 MHz, CDCl_3) δ 197.1, 192.6, 169.6, 169.0, 164.3, 157.5, 105.8, 105.1, 97.0, 90.9, 89.2, 81.6, 56.8, 56.3, 39.8, 36.1, 32.1 (2C), 23.7 (2C) 14.2. HRMS (ESI^+) calcd for $[\text{C}_{21}\text{H}_{24}\text{ClO}_6]^+$ 407.1261, found 407.1262.

General Procedure for the Synthesis of Enol Ethers by Addition–Elimination (19–25). To a solution of **18** (0.65 mmol, 1 equiv) in 1,4-dioxane (3 mL, 0.2 M) was added the desired alcohol (1.30 mmol, 2 equiv) and DBU (1.63 mmol, 2.5 equiv). The mixture was heated to 100 °C and stirred for 12 h. The mixture was then cooled to 20 °C, and excess reagent was quenched with sat. aq. NH_4Cl (30 mL). The aqueous phase was extracted with EtOAc (3 \times 30 mL) and the combined organic phases were dried (MgSO_4) and then concentrated. The residue was purified by column chromatography (heptane:EtOAc 3:2) affording the product. When possible the product was recrystallized from EtOAc/heptane.

(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(6'-methyl-2'-(2-phenylethoxy)-cyclohex-2'-ene-4'-one) (22). Yield: 199 mg (86%); R_f (toluene: CH_2Cl_2 :EtOAc 1:1:1): 0.50. ^1H NMR (300 MHz, CDCl_3) δ 7.20–7.12 (3H, m), 7.03–6.95 (2H, m), 6.13

(1H, s), 5.49 (1H, s), 4.06 (3H, s), 3.97 (3H, s), 3.95–3.84 (2H, m), 3.02 (1H, dd, J = 16.5, 13.5 Hz), 2.89–2.75 (3H, m), 2.40 (1H, dd, J = 16.5, 4.6 Hz), 0.94 (3H, d, J = 6.7 Hz). ^{13}C NMR (75 MHz, CDCl_3) 197.0, 192.5, 169.8, 169.5, 164.5, 157.6, 137.2, 128.8 (2C), 128.3 (2C), 126.4, 105.1, 105.0, 97.1, 90.7, 89.4, 69.9, 57.0, 56.3, 39.9, 36.1, 34.7 14.1. HRMS (ESI^+) calcd for $[\text{C}_{24}\text{H}_{24}\text{ClO}_6]^+$ 443.1261, found 443.1264.

(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(6'-methyl-2'-(4-methylbenzyloxy)-cyclohex-2'-ene-4'-one) (23). Yield: 201 mg (54%) (white crystals); R_f (EtOAc:heptane 5:1): 0.45; mp: 176–178 °C. IR (KBr, cm^{-1}) 1709, 1664. ^1H NMR (300 MHz, CDCl_3) δ 7.11–7.04 (4H, m), 6.09 (1H, s), 5.58 (1H, s), 4.87 (1H, d, J = 12.2 Hz), 4.76 (1H, d, J = 12.2 Hz), 4.01 (3H, s), 3.95 (3H, s), 3.04 (1H, dd, J = 16.5, 13.4 Hz), 2.85 (1H, ddq, J = 13.2, 4.5, 6.6 Hz), 2.41 (1H, dd, J = 16.4, 4.4 Hz), 2.30 (3H, s), 0.97 (3H, d, J = 6.6 Hz). ^{13}C NMR (75 MHz, CDCl_3) δ 197.1, 192.7, 169.9, 169.8, 164.7, 157.9, 138.2, 131.8, 129.5 (2C), 127.0 (2C), 106.2, 105.8, 97.1, 91.0, 89.7, 71.0, 57.2, 56.6, 40.2, 36.7, 21.4, 14.5. HRMS (ESI^+) calcd for $[\text{C}_{24}\text{H}_{24}\text{ClO}_6]^+$ 443.1261, found 443.1273.

(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(2'-(4-biphenylmethoxy)-6'-methyl-cyclohex-2'-ene-4'-one) (24). Yield: 54 mg (19%); R_f (toluene: CH_2Cl_2 :heptane 2:2:1): 0.32. IR (KBr, cm^{-1}) 1704, 1662. ^1H NMR (300 MHz, CDCl_3) δ 7.58–7.50 (4H, m), 7.46–7.40 (2H, m), 7.37–7.31 (1H, m), 7.28–7.23 (2H, m), 6.10 (1H, s), 5.63 (1H, s), 4.97 (1H, d, J = 12.4 Hz), 4.85 (1H, d, J = 12.4 Hz), 4.01 (3H, s), 3.96 (3H, s), 3.07 (1H, dd, J = 16.5, 13.4 Hz), 2.88 (1H, ddq, J = 13.4, 4.6, 6.6 Hz), 2.45 (1H, dd, J = 16.5, 4.6 Hz), 1.00 (3H, d, J = 6.6 Hz). ^{13}C NMR (50 MHz, CDCl_3) δ 197.0, 192.4, 169.5 (2C), 164.5, 157.7, 141.0, 140.4, 133.6, 128.7 (4C), 127.2, 127.0 (4C), 105.9, 105.3, 97.2, 90.7, 89.4, 70.4, 56.9, 56.3, 40.0, 36.3, 14.2. HRMS (ESI^+) calcd for $[\text{C}_{29}\text{H}_{26}\text{ClO}_6]^+$ 505.1418, found 505.1421.

(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(2'-(1-adamantylmethoxy)-6'-methyl-cyclohex-2'-ene-4'-one) (25). Yield: 5 mg (2%); R_f (toluene: CH_2Cl_2 :EtOAc 3:3:1): 0.16. ^1H NMR (300 MHz, CDCl_3) δ 6.05 (1H, s), 5.37 (1H, s), 3.96 (3H, s), 3.91 (3H, s), 3.26 (1H, d, J = 9.2 Hz), 3.13 (1H, d, J = 9.2 Hz), 3.03 (1H, dd, J = 16.6, 13.6 Hz), 2.87–2.71 (1H, m), 2.36 (1H, dd, J = 16.6, 4.8 Hz), 1.83–1.75 (3H, m), 1.63–1.50 (4H, m), 1.45–1.28 (6H, m), 1.20–1.13 (2H, m), 0.95 (3H, d, J = 6.7 Hz). ^{13}C NMR (50 MHz, CDCl_3) δ 197.1, 192.8, 170.3 (2C), 164.5, 157.7, 104.2 (2C), 97.4, 91.3, 89.3, 78.6, 57.0, 56.4, 40.3, 38.8 (3C), 36.8 (3C), 35.7, 33.5, 27.9 (3C), 14.3. HRMS (ESI^+) calcd for $[\text{C}_{27}\text{H}_{32}\text{ClO}_6]^+$ 487.1887, found 487.1888.

(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(2'-benzyloxy-6'-methylcyclohex-2'-ene-4'-one-4'-oxime) (29). To a solution of **20** (0.20 mmol, 1.0 equiv) in EtOH (5 mL, 0.03M) and DMSO (2.5 mL, 0.03M) was added hydroxylamine hydrochloride (0.70 mmol, 3.5 equiv) and sodium acetate (0.86 mmol, 4.3 equiv). The mixture was stirred at 75 °C for 24 h, allowed to reach 20 °C, and diluted with CH_2Cl_2 (20 mL). The mixture was washed with distilled water (2 \times 15 mL) and then brine (15 mL). The organic phase was dried (MgSO_4) and concentrated. The crude mixture was purified by column chromatography (toluene: CH_2Cl_2 :EtOAc 2:2:1) to afford the desired product.

Yield: 167 mg (82%) (white needles); R_f (EtOAc:heptane 5:1): 0.39 and 0.36; mp: 139–141 °C. IR (KBr, cm^{-1}) 1706, 1614. ^1H NMR (300 MHz, CDCl_3) δ 8.63 (1H, s), 7.30–7.13 (5H, m), 6.36 (0.5H, s), 6.07 (1H, s), 6.68 (0.5H, s), 4.99–4.71 (2H, m), 3.99 (3H, s), 3.93 (3H, s), 3.14 (0.5H, dd, J = 16.6, 4.7 Hz), 3.04 (0.5H, dd, J = 15.0, 13.2 Hz), 2.73 (0.5H, dd, J = 16.6, 13.0 Hz), 2.69–2.51 (1H, m), 2.42 (0.5H, dd, J = 15.0, 4.2 Hz), 0.98 (1.5H, d, J = 6.6 Hz), 0.97 (1.5H, d, J = 6.8 Hz). ^{13}C NMR (75 MHz, CDCl_3) δ 194.0 (0.5C), 193.8 (0.5C), 159.8 (0.5C), 157.4, 157.2 (0.5C), 155.1 (0.5C), 151.8 (0.5C), 135.7 (0.5C), 135.5 (0.5C), 128.3 (2C), 127.7 (0.5C), 127.6 (0.5C), 126.6, 126.5, 105.7, 100.3 (0.5C), 97.0, 93.7 (0.5C), 91.5 (0.5C), 91.4 (0.5C), 89.1, 70.0 (0.5C), 69.7 (0.5C), 56.9, 56.2, 36.4 (0.5C), 35.2 (0.5C), 30.9 (0.5C), 25.5 (0.5C), 14.4 (0.5C), 14.3 (0.5C). HRMS (ESI^+) calcd for $[\text{C}_{25}\text{H}_{22}\text{ClNO}_6]^+$ 444.1214, found 444.1204.

(2S,6'R,E)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-ene-4'-one-4'-dimethylhydrazine) (**30**). To a solution of griseofulvin (1.0 g, 2.83 mmol) in toluene (28.3 mL) was added *N,N*-dimethylhydrazine (0.9 mL, 11.32 mmol) and 90% aq acetic acid (0.5 mL, 8.66 mmol). The mixture was heated to 50 °C for 24 h and cooled to 20 °C. The mixture was diluted with diethyl ether (100 mL), washed with sat. aq NaHCO₃ (50 mL) and brine (50 mL). The combined aqueous phases were extracted with diethyl ether (50 mL), and the combined organic phases were dried (Na₂SO₄) and concentrated. The residue was purified by column chromatography (toluene:CH₂Cl₂:EtOAc 1:1:2) to yield compound **30**. Yield: 208 mg (19%) (orange needles); *R*_f (EtOAc:heptane 5:1): 0.22; mp: 118–120 °C. IR (KBr, cm⁻¹) 1708, 1613. ¹H NMR (300 MHz, CDCl₃) δ 6.11 (1H, s), 5.67 (1H, s), 4.02 (3H, s), 3.97 (3H, s), 3.56 (3H, s), 3.13 (1H, dd, *J* = 16.2, 4.5 Hz), 2.77 (1H, dd, *J* = 16.2, 12.9 Hz), 2.60 (1H, ddq, *J* = 12.8, 4.7, 6.6 Hz), 2.53 (6H, s), 0.94 (3H, d, *J* = 6.7 Hz). ¹³C NMR (75 MHz, CDCl₃) δ 194.4, 169.7, 164.5, 162.7, 160.0, 157.7, 105.8, 103.3, 97.3, 91.9, 89.4, 57.2, 56.5, 56.2, 47.5 (2C), 36.1, 29.4, 14.6. HRMS (ESI⁺) calcd for [C₁₉H₂₄ClN₂O₅]⁺ 395.1374, found 395.1374.

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Supporting Information Available: Full experimental data for compounds **2**, **4–8**, **10–15**, **18–21**, **26–28**, and **31–35** as well as copies of NMR spectra for compounds **2** and **4–35**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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CHAPTER 4

Disparate SAR Data from Griseofulvin Analogs Tested Against the Dermatophytes *Trichophyton mentagrophytes*, *T. rubrum* and MDA-MB-231 Cancer Cells

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The supporting information for Chapter 4 can be seen in Appendix D

Disparate SAR Data of Griseofulvin Analogs for the Dermatophytes *Trichophyton mentagrophytes*, *T. rubrum* and MDA-MB-231 Cancer Cells

Mads H. Rønneest,^{a,b} Marc S. Raab,^{c,d} Simon Anderhub,^d Sven Boesen,^a Alwin Krämer,^d Thomas O. Larsen,^{a*} Mads H. Clausen^{b*}

^aCenter for Microbial Biotechnology, Department of Systems Biology, Søtofts Plads, Building 221, DK-2800 Kgs. Lyngby, Denmark; ^bDepartment of Chemistry, Technical University of Denmark, Kemitorvet, Building 201, DK-2800 Kgs. Lyngby, Denmark; ^cMax-Eder Group "Experimental Therapies for Hematologic Malignancies", German Cancer Research Center (DKFZ) and Department of Internal Medicine V, University of Heidelberg, Im Neuenheimer Feld 280 (TP4) 69120 Heidelberg, Germany. ^dClinical Cooperation Unit Molecular Hematology/Oncology, German Cancer Research Center (DKFZ) and Department of Internal Medicine V, University of Heidelberg, Im Neuenheimer Feld 280 (TP4) 69120 Heidelberg, Germany.

Correspondence e-mails: Thomas O. Larsen: tol@bio.dtu.dk; Mads H. Clausen: mhc@kemi.dtu.dk

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Abstract

Griseofulvin (**1**) and 53 analogs of this compound have been tested against the pathogenic dermatophytes *Trichophyton rubrum* and *T. mentagrophytes* as well as against the breast cancer cell line MDA-MB-231. The modifications to griseofulvin include the 4, 5, 6, 2', 3' and 4' positions. The SAR of the griseofulvin analogs towards the two fungi followed the same trend with the majority being less active than griseofulvin and none had more than twice the potency of the parent compound. A comparison of the anti-fungal and the anti-cancer SAR revealed distinct differences, as the majority of analogs showed increased activity against the cancer cell line MDA-MB-231, highlighted by 2'-benzyloxy-2'-demethoxy-griseofulvin, which showed low activity against both fungi, but was among the most potent compounds against MDA-MB-231 cancer cells. Tubulin has been proposed as the target of griseofulvin in both fungal and mammalian cells, but the differences revealed by this SAR study strongly suggest that the mode-of-action of the compound class towards fungi and mammalian cancer cells is different.

Introduction

Griseofulvin (**1**, see Figure 1) was one of the first anti-fungal natural products isolated¹ from filamentous fungi and has been known as an anti-fungal agent for decades.^{2,3} The compound was until the approval of Lamisil[®] by the US Food and Drug Administration⁵ in 2007, the only drug available for treatment of tinea capitis,⁶ a superficial fungal skin infection caused by dermatophytes, which predominantly affects children.⁷ Upon administration to man griseofulvin accumulates in the skin (*Stratum corneum*) where it presumably binds to keratin.⁸ The mode of action is still not determined but tubulin binding has been proposed.^{9,10}

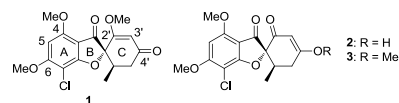


Figure 1. The structure of griseofulvin (**1**), griseofulvic acid (**2**) and isogriseofulvin (**3**). The rings A, B and C as well as the positions modified in this study in **1** are shown.

More than four hundred griseofulvin analogs have been reported since its discovery and the activity of these over three hundred of these have been compared with griseofulvin (**1**) against six dermatophytes in a study by Crosse et al.¹¹ The curling of hyphae in *Botrytis allii* were also tested, but this phenotype did not correlate with the growth inhibitory effect of the analogs.¹¹ Griseofulvin analogs with modifications at positions 4, 6, 2' and 3' as well as isogriseofulvin analogs with modifications at the 4, 6, 3' and 4' positions were tested. No 2' analogs excelled in growth inhibition of the dermatophytes,¹¹ but elongation did increase the curling of hyphae with the optimal analogs the being 2'-propoxy (**46**) and 2'-butoxy analogs.¹² Most griseofulvin analogs tested showed increased potency against some dermatophytes, but exhibiting lower activity against others.

Position 5 has been functionalized with nitro (**10**) and amine (**9**) groups rendering the analogs inactive at relevant concentrations against four dermatophytes.¹³ An ethoxycarbonyl group has also been introduced in this position and the analog found to be inactive.¹⁴ The 4' position has also been examined with the 4' alcohol analog (**50**) being inactive and the 4' oxime (**36**) being seven fold

less potent compared to **1**, with both analogs tested against *Microsporum gypseum*.¹⁵ Although the initial isolation of **1** was completed in 1939¹ both anti-cancer¹⁶⁻¹⁹ and anti-viral²⁰ properties of griseofulvin have been discovered recently. Three analogs tested for the former by Oda et al.²¹ against chinese hamster V79 cells showed increased cytotoxicity with 2'-propoxy-2'-demethoxy-griseofulvin being the most potent (**46**, IC₅₀ 0.7 μ M; **1**, 8 μ M) and it was proposed that additional structural modifications at the 2' position could enhance activity further. This was supported by ourselves in a whole cell phenotype-based anti-cancer assay for spindle multipolarity induction, where increased activity was seen for analogs with modifications in the 2' position, with the 2'-benzyloxy-2'-demethoxy-griseofulvin analog (**17**) being the most potent compound tested.²² Multiple papers state that griseofulvin arrests several cancer cell lines in G₂/M phase of the cell cycle.¹⁷⁻¹⁹ Several investigators have proposed tubulin as the main target for griseofulvin although for mammalian cells this proposition is not undisputed.^{9,17,18,20,21,23,24} Recently, Panda et al.²⁵ proposed two griseofulvin binding sites on tubulin using molecular docking studies and similar to Oda²¹ reported that microtubule dynamics were disrupted by **1**. Using the hepatitis C virus-1b cell culture system Huh7/Rep-Feo, Jin et al. reported that G₂/M phase arrest in infected cells was induced by griseofulvin (**1**).²⁰ It was speculated that the effect was due to interaction with microtubule polymerization.²⁰ Griseofulvin exhibits activity against fungi, mammalian cancer cells as well as suppressing RNA replication by the hepatitis C virus, with tubulin having been proposed to be involved in all three cases. Tubulins are very conserved within different eukaryotic cell types,²⁴ and most of the variation among different tubulin isoforms is found in the amino acids near the C-terminus, which form a ridge on the surface of microtubules. Therefore, variations among different isoforms are expected to affect primarily the association of accessory proteins with the surface of microtubules, rather than microtubule polymerization *per se*. In case tubulin is the sole target of griseofulvin in both fungi and mammalian cells, the activity profile of an array of analogs against these cell types should be similar. To test this hypothesis we decided to carry out an SAR study of griseofulvin analogs. This is to the best of our knowledge the first study of griseofulvin (**1**) and analogs thereof, which compares anti-fungal and anti-cancer SAR from growth inhibition assays.

Griseofulvin (**1**) and 54 griseofulvin analogs (11 reported for the first time here) have been tested in an anti-fungal assay against *T. mentagrophytes* and *T. rubrum*, two dermatophytes causing *tinea capitis*.⁷ All compounds were also tested in an anti-cancer assay against the human cell line MDA-MB-231 (breast adenocarcinoma), which was chosen because this cell line represents a common cancer type and is known to harbor supernumerary centrosomes

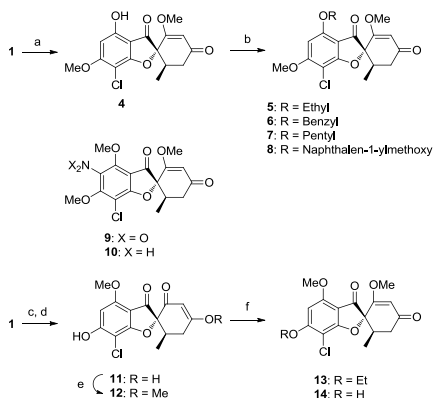
which are regularly clustered into a bipolar mitotic spindle array in a high percentage of cells.²⁶ Analogs with alterations at the 4, 5, 6, 2', 3' and 4' positions have all been synthesized from commercially available griseofulvin in one to five synthetic steps.

Results and Discussion

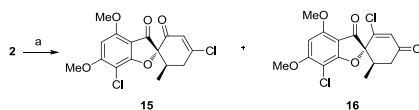
Chemistry. The 4-phenol (**4**) was synthesized from **1** by treatment with freshly prepared MgI₂, procured by sonication of Mg and I₂ in Et₂O/toluene, affording **4** in 99% yield, an improvement on prior methods (Scheme 1).^{22,27} Alkylation of **4** to synthesize **5** and **6** has previously been described.^{22,27} Position 4 analogs **7** and **8** were prepared from **4** with Ag₂O and the appropriate alkyl bromide in dioxane as solvent.²⁷ The syntheses of **9** and **10** have already been described.^{22,28}

For the preparation of **11**, **12** and **13** see Rønneest et al.²⁹ and Arkley et al.²⁷ Compound **12** was alkylated using Ag₂O and EtBr followed by repeated solvolysis in MeOH with CSA to afford **14**. The dichloro analogs **16** and **15** were synthesized using **2**,³⁰ POCl₃, LiCl and dioxane, a modification of a known method (Scheme 2).³¹ The compounds **17-33** were synthesized by 1,4 addition of the corresponding alcohol to **16** using either NaH or DBU as base (Scheme 3).³¹ The isogriseofulvin analogs **3** and **35** were synthesized in the same manner but from **15**. Compound **3** has previously been prepared by treating **2** with excess diazomethane yielding both **1** and **3**.³² Compounds **26** and **38** have been reported in a patent,³³ which is also the case for **19**, **21** and **22**.³⁴ The dimer **34**, which was conveniently synthesized together with **22** by a second 1,4-addition of **22** to **16**, has previously been published in a Japanese patent.³⁵

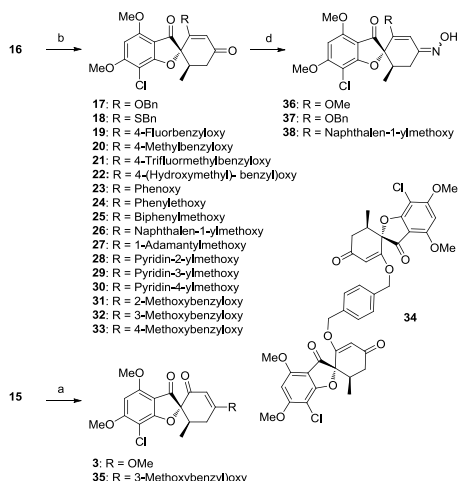
The analogs with an oxime functionality **36**, **37** and **38** were synthesized from the corresponding ketones (**1**, **17** and **26**) using hydroxylamine hydrochloride in ethanol and DMSO.^{13,22} Isogriseofulvin (**39-44**) and griseofulvin analogs (**45-49** and **17**) were synthesized simultaneously in pairs by solvolysis with the respective alcohols and catalytic CSA and subsequently separated by chromatography (Scheme 4). Analogs **50-55** were synthesized according to published procedures.²² The fungal secondary metabolite geodin (**56**) was recently isolated from *Aspergillus terreus*.³⁶ Please see the supporting information for ¹H and ¹³C NMR spectra for all compounds and experimental for compounds **2**, **3**, **15**, **39-41** and **44**.



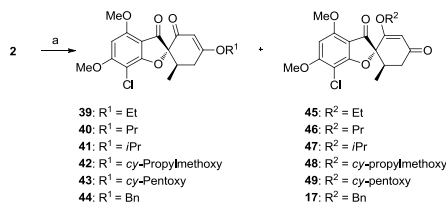
Scheme 1. (a) MgI_2 , toluene, Et_2O ; (b) RBr , Ag_2O , dioxane; (c) HOAc , 2 M H_2SO_4 ; (d) 0.66 M NaOH ; (e) MeOH , CSA ; (f) MeOH , CSA (**13**) or EtBr , Ag_2O , dioxane; then MeOH , CSA (**14**).



Scheme 2. (a) LiCl , POCl_3 , dioxane.



Scheme 3. (a) ROH , DBU , THF or dioxane. (b) hydroxylamine, NaOAc , EtOH , DMSO .



Scheme 4. (a) ROH , CSA , dioxane (**17**, **42-44**, **48**, and **49**).

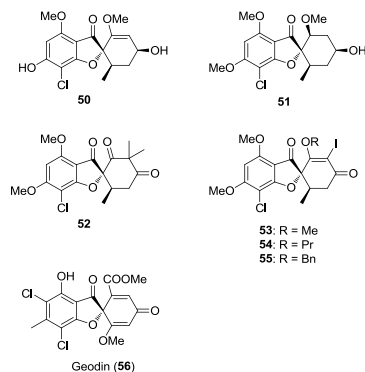


Figure 2. The analogs **50-55** were prepared according to literature procedures.²² Geodin (**56**) was isolated as previously described.³⁶

Assays. Griseofulvin (**1**), geodin (**56**) and 53 griseofulvin analogs covering variations on six positions (see Figure 1) were tested against two dermatophytes (*T. rubrum* and *T. mentagrophytes*) and against the breast cancer cell line MDA-MB-231 in a cytotoxicity assay. For all the test results the following definitions were used: if no activity was observed at 50 μM a given compound was deemed *inactive*; if activity was observed but 50% inhibition was not reached at 50 μM a given compound is described as having low activity and no IC_{50} value is calculated (see supporting information for examples). The IC_{50} of griseofulvin (**1**) was determined to be $0.38 \pm 0.048 \mu\text{M}$ against the *T. rubrum* isolate and $0.058 \pm 0.018 \mu\text{M}$ against the *T. mentagrophytes* isolate. All IC_{50} values and 95% confidence intervals are given in Table 1.

Anti-fungal Structure-Activity Relationship for *T. rubrum* and *T. mentagrophytes*. All compounds in the position 4 series of **4-8** were less active than griseofulvin (**1**) against the two fungi and **8** was completely inactive. For *T. rubrum* **4** and **6** were inactive, **7** had low activity and **5** was, with an IC_{50} of 2.0 μM , approximately five times less potent than griseofulvin. For *T. mentagrophytes* analog **4**

showed low activity and the rest were less potent than **1**: **5** (0.29 μ M), **6** (0.25 μ M) and **7** (0.17 μ M). The activity of **6** has previously been reported against a number of dermatophytes (*Epidermophyton floccosum*, *Microsporium canis*, *T. interdigitale*, *T. persicolor*, *T. mentagrophytes* and *T. rubrum*) and found to be less active than **1** against all of them.¹¹

The nitro (**9**) and amine (**10**) position 5 analogs were inactive against both fungi. Compound **10** has also been reported as inactive, but **9** has been reported as weakly active with inhibition for both fungi starting at around 25 μ M.¹³ Other position 5 analogs (ethoxycarbonyl,¹⁴ methoxy, methyl or chloro³⁷) have been reported to have lower activity than **1** against dermatophytes.

Whereas the position 6 phenols **13** and **11** were inactive against both fungi, the 6-ethyl analog (**14**) had an IC₅₀ of 1.3 μ M against *T. rubrum* and was as active as griseofulvin (**1**) against *T. mentagrophytes* with an IC₅₀ of 0.062 μ M, which is in accordance with the literature.¹¹

The 2' series is the most extensively studied due to the increased activity observed in the anti-cancer phenotype-based assay¹⁹ by these analogs. For *T. rubrum* elongation to 2'-ethyloxy (**45**, 0.69 μ M) and 2'-propyloxy (**46**, 0.62 μ M) lowered the activity twofold, confirming the findings of Crosse et al.¹¹ The bulkier 2'-isopropyloxy (**47**, 3.1 μ M) was ten times less potent. Increasing bulkiness through 2'-cyclopropylmethoxy (**48**, 1.5 μ M), cyclopentyloxy (**49**, 2.5 μ M) and benzyloxy (**17**, 1.1 μ M) enol ethers did not reveal a trend but all three were less active towards *T. rubrum* than **1**. No correlation between the size of the 2' substituent and the activity was seen for *T. mentagrophytes* with **46** (0.050 μ M) retaining the activity of **1** and IC₅₀ values of 0.25 μ M and 0.11 μ M for **45** and **47**, respectively. Both **45** and **46** have been reported to retain the activity of **1** against *T. mentagrophytes* by Crosse et al.¹¹ Analog **48** (0.060 μ M) had the same activity as **1**, but **17** (0.26 μ M) and **49** (0.24 μ M) were about four times less potent.

The activities of **17**, **45** and **46** have been reported against *T. mentagrophytes* and *T. rubrum* and all showed lower activity when tested against the latter.¹¹ Lower activity than **1** towards *T. mentagrophytes* was also reported for **17**, but **45** and **46** had similar potency.¹¹ The three compounds were also tested against *Epidermophyton floccosum*, *Microsporium canis*, *T. interdigitale* and *T. persicolor* displaying the same trends, with **17** having lower activity and **45** and **46** retaining the activity of **1**.¹¹

The rest of the 2' series includes bulkier analogs (**25-27**), para-substituted variations of **17** (**19-22**), two sets of ortho-, meta- and para-analogs (**28-30** and **31-33**), modification of the linker part (**23** and **24**) and a vinyl sulfide analog **18**.

Of the bulkier 2' analogs the naphthalen-1-ylmethoxy **26** was the only active analog and **25** and **27** were inactive against both fungi. With IC₅₀ values of 2.6 μ M (*T. rubrum*) and 0.25 μ M (*T. mentagrophytes*) compound **26** is three and four times less potent than **1**, respectively.

The three 2' pyridine analogs (**28-30**) had low activity towards *T. rubrum* and **30** also showed low activity towards *T. mentagrophytes*. Compounds **29** and **28** had IC₅₀ values of 0.35 μ M and 1.3 μ M, meaning they were approximately six and 22 times less potent towards *T. rubrum* compared to **1**. The series with ortho-, meta- and para-methoxy groups revealed **33** as inactive against both fungi, **31** with low activity towards *T. rubrum* and an IC₅₀ of 0.70 μ M against *T. mentagrophytes*. The meta-substituted analog **32** (0.96 μ M) was threefold less active against *T. rubrum* compared to **1** and displayed a twofold decrease in activity against *T. mentagrophytes* with an IC₅₀ of 0.14 μ M.

The 2'-phenoxy analog (**23**) had IC₅₀ values of 6.1 μ M against *T. rubrum* and 1.6 μ M against *T. mentagrophytes*, while the phenylethoxy analog (**24**) had low activity against *T. rubrum* and an IC₅₀ of 0.28 μ M against *T. mentagrophytes*. Lower activity for **23** has previously been reported.¹¹ The vinyl sulfide analog **18** (1.3 μ M) was as active as **17** against *T. rubrum* but against *T. mentagrophytes* the IC₅₀ (0.058 μ M) was equal to that of griseofulvin (**1**).

The 3'-dimethyl analog **52** was inactive against both fungi. The series of 3' analogs include three 3'-iodo analogs with 2' modifications, 2'-methoxy (**53**), 2'-ethoxy (**54**) and 2'-benzyloxy (**55**). The 3'-iodo griseofulvin (**53**) was inactive against *T. rubrum* and had low activity towards *T. mentagrophytes* in accordance with Crosse et al.¹¹ The 2'-benzyloxy (**55**) analog was inactive against *T. rubrum* and close to twenty times less potent (1.1 μ M) than **1** against *T. mentagrophytes*. Analog **55** was also four times less potent than **17**, which has no 3' iodo group. The propoxy (**54**) analog showed activity against both fungi (*T. rubrum* 1.6 μ M and *T. mentagrophytes* 0.23 μ M) but was less potent than both **1** and the analog **46**, which does not contain iodide. Analog **54** has been reported to be the most potent at inducing curling of hyphae, but less active against all dermatophytes spare one.¹¹

The introduction of an oxime functionality on the parent compounds **1**, **17** and **26** afforded the analogs **36**, **37** and **38** and lowered the activity towards both fungi compared to the parent compounds (**38** was inactive against both fungi altogether). The IC₅₀ values for **36** were 1.3 μ M against *T. rubrum* and 0.19 μ M against *T. mentagrophytes* which is in accordance with previously published data.¹³ Analog **36** has also been reported active against *M. gypseum*.¹⁵ Compound **37** had IC₅₀ values of 7.9 μ M against *T. rubrum* and of 0.30 μ M against *T. mentagrophytes*, twenty and three times less potent than **1**, respectively. The 4' alcohol **50** exhibited low activity towards both fungi which was not in agreement with prior work as it has been reported that **50** causes 100% growth inhibition of *T. rubrum* and visible inhibition against *T. mentagrophytes* at 28 μ M.¹³ Compound **50** has however been tested inactive against *M. gypseum*.¹⁵

The series including isogriseofulvin (**3**) and isogriseofulvin analogs **35** and **39-44** was inactive against *T. rubrum* and showed low activity against *T. mentagrophytes*. This is in

GF#	T. rubrum (μ M)	\pm STD IC ₅₀ (μ M)	T. menta- grophytes (μ M)	\pm STD IC ₅₀ (μ M)	Cytotox (μ M)	\pm STD IC ₅₀ (μ M)
1	0.38	0.05	0.058	0.02	18	4
2	Inactive	Inactive	Low activity	-	Inactive	Inactive
3	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
4	Inactive	Inactive	Low activity	-	Inactive	Inactive
5	2.0	0.7	0.29	0.1	Inactive	Inactive
6	Inactive	Inactive	0.25	0.009	20	4
7	Low activity	-	0.17	0.02	Low activity	-
8	Inactive	Inactive	Inactive	Inactive	17	2
9	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
10	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
11	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
13	Inactive	Inactive	Inactive	Inactive	Low activity	-
14	1.3	0.1	0.062	0.02	36	3
15	Inactive	Inactive	Inactive	Inactive	1.0	0.1
16	Low activity	-	Low activity	-	3.2	0.3
17	1.1	0.2	0.26	0.1	2.1	0.2
18	1.3	0.4	0.058	0.02	4.7	0.7
19	0.83	very wide	0.041	0.01	6.5	1
20	1.0	0.2	0.24	0.1	1.8	0.3
21	1.5	0.2	0.15	0.1	7.0	0.9
22	Low activity	-	1.7	1	2.8	0.6
23	6.1	7	1.6	3	7.2	1
24	Low activity	-	0.28	0.2	17	2
25	Inactive	Inactive	Inactive	Inactive	5.8	1
26	2.6	0.3	0.25	0.09	13	2
27	Inactive	Inactive	Inactive	Inactive	4.7	0.5
28	Low activity	-	1.3	3	32	6
29	Low activity	-	0.35	0.2	10	2
30	Low activity	-	Low activity	-	19	2
31	Low activity	-	0.70	0.6	11	3
32	0.96	0.4	0.14	0.07	8.1	2
33	Inactive	Inactive	Inactive	Inactive	Low activity	-
34	Inactive	Inactive	Inactive	Inactive	8.5	2
35	Inactive	Inactive	Low activity	-	25	5
36	1.3	0.5	0.19	0.1	12	2
37	7.9	0.9	0.30	0.2	1.4	0.3
38	Inactive	Inactive	Inactive	Inactive	8.3	0.8
39	Inactive	Inactive	Low activity	-	Low activity	-
40	Inactive	Inactive	Low activity	-	43	6
41	Inactive	Inactive	Low activity	-	48	10
42	Inactive	Inactive	Low activity	-	52	10
43	Inactive	Inactive	Low activity	-	27	4
44	Inactive	Inactive	Low activity	-	Low activity	-
45	0.69	0.2	0.25	0.08	13	3
46	0.62	0.09	0.050	0.02	16	5
47	3.1	1	0.11	0.05	11	3
48	1.5	0.3	0.060	0.02	9.4	2
49	2.5	0.8	0.24	0.09	3.2	0.5
50	Low activity	-	Low activity	-	Inactive	Inactive
51	Low activity	-	3.0	1	Inactive	Inactive
52	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
53	Inactive	Inactive	Low activity	-	22	1
54	1.6	0.4	0.23	0.08	8.0	1
55	Inactive	Inactive	1.1	0.5	Low activity	-
56	Inactive	Inactive	Inactive	Inactive	9.9	1

Table 1. All available IC₅₀ values from the screen. If no activity was observed at 50 μ M a given compound is deemed *inactive*. If activity was observed, but 50 % inhibition was not reached at 50 μ M a given compound is described as having low activity and the IC₅₀ value is not calculated.

accordance with the literature, where **3** and **39** have been reported to be less active than griseofulvin against a number of dermatophytes (*E. floccosum*, *M. canis*, *T. interdigitale*, *T. persicolor*, *T. mentagrophytes* and *T. rubrum*).¹¹ Griseofulvic acid (**2**) was inactive against *T. rubrum* and had low activity against *T. mentagrophytes* contrary to prior work, which reported **2** to be inactive towards *T. mentagrophytes* but having visible growth inhibition at 30 μ M against *T. rubrum*.¹³ The griseofulvin dimer **34** was inactive against both fungi. The dichloro analog **16** showed low activity towards both fungi while **15** was inactive against both. The reduced analog **51** had low activity against *T. rubrum* and an IC_{50} of 3.0 μ M against *T. mentagrophytes*, which is 50 times less potent compared to **1**. Geodin (**56**) was inactive against both fungi.

Of the 55 compounds tested, 27 analogs were inactive or exhibited low activity for both fungi (see Table 1). Nine of the compounds were active against both fungi within the same order of magnitude compared to griseofulvin (**17**, **20**, **21**, **23**, **26**, **32**, **36**, **49** and **54**).

There were however some compounds that fared differently against the two fungi. The compounds **6**, **7**, **22**, **24**, **28**, **29**, **31**, **51** and **55** were all inactive or had low activity towards *T. rubrum* but had IC_{50} values in the range of 0.25–3.0 μ M against *T. mentagrophytes*. For other analogs (**14**, **18**, **19**, **37**, **47** and **48**) the activity against *T. mentagrophytes* was similar to that of **1** but against *T. rubrum* it was significantly lower. This is in accordance with earlier observations by Crosse et al., demonstrating that a given analog was more active against some fungi but less active against others.¹¹

Anti-cancer Structure-Activity Relationship. Looking at the IC_{50} values for the 16 analogs (**1**, **17**, **18**, **20**, **23–25**, **27**, **36**, **37**, **45–49** and **54**) tested active in both the multipolarity assay¹⁹ and the cytotoxicity based assay there was good correlation between the data with an R^2 of 0.70 (see supporting information).

The IC_{50} of griseofulvin (**1**) was determined to be 23 ± 5 μ M (20 ± 1 μ M in the phenotype-based spindle multipolarity assay²³), while an IC_{50} of 25 ± 4 μ M against HeLa cells was found by Panda et al.¹⁸ In the position 4 series, **4**, **5** and **7** were all inactive but **6** and **8** harboring aromatic moieties had activities similar to griseofulvin (**1**) with IC_{50} values of 20 (**6**) and 17 μ M (**8**). This indicates a mode of action that does not involve induction of multipolar mitosis as **6** was inactive in the multipolarity assay. Both the position 5 analogs **9** and **10** were inactive in the cytotoxicity assay as they were in the assay for multipolarity induction.

The 6-phenol griseofulvic acid analog **11** was inactive and the 6-phenol griseofulvin analog (**13**) showed low activity. The 6-ethyl griseofulvin (**14**) analog was approximately 50% less active than griseofulvin. The two dichloro analogs **15** and **16** were some of the most cytotoxic among the tested analogs with IC_{50} values of 1.0 and 3.2 μ M, respectively.

When elongating the 2' position from the parent methoxy (**1**) to ethoxy (**45**) and propoxy (**46**) and then further

increasing the bulkiness with isopropoxy (**47**), cyclopentoxy (**49**) and benzyloxy (**17**) the activity increased through the series (see Figure 3), plateauing with **17** and **49** at 2.1 and 3.2 μ M, respectively. The same trend was seen when these compounds were tested in the phenotype-based multipolarity assay. Moving from the benzyloxy derived analogs to even bulkier groups like the naphthalen-1-ylmethoxy (**26**, 13 μ M), biphenylmethoxy (**25**, 5.8 μ M) and 1-adamantylmethoxy (**27**, 4.7 μ M) analogs, the activity did not increase further. The three compounds were still more potent than griseofulvin though, which was also observed for **25** and **27** in the phenotype-based assay.

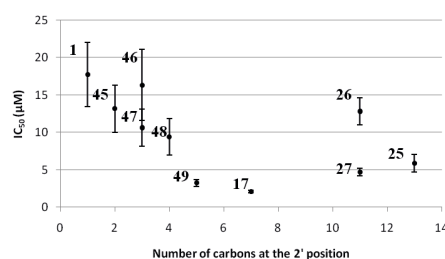


Figure 3. The activity increases with the number of carbon atoms at the 2' position from griseofulvin itself to the 2' ethoxy (**45**), propoxy (**46**), isopropoxy (**47**) and cyclopentylmethoxy (**48**) before leveling out with the cyclopentoxy (**49**) and benzyloxy (**17**) analogs. The bulkier analogs **25**, **27** and **26** were less active than the benzyloxy analog (**17**).

A number of variations of **17** have been tested (**18–24**, **28–30** and **31–33**) and although all except **33** (low activity), **28** (32 μ M) and (**24**, 17 μ M; **30**, 19 μ M) were more active than **1**, only **20** was as active as **17** with an IC_{50} of 1.8 μ M. The difference in activity between the phenoxy (**23**, 7.2 μ M) and phenylethoxy (**24**, 17 μ M) analogs was less pronounced in the cytotoxicity assay than in the multipolarity assay with two and a half orders of magnitude compared to the approximately seven-fold difference in the phenotype-based assay. The dimer (**34**) was about twice as active as **1** with an IC_{50} value of 8.5 μ M.

The introduction of an oxime functionality at the 4' position (**36**) increased potency twofold to 12 μ M, an increase in activity that was also seen in the phenotype-based assay. Introducing the oxime to analogs **17** and **26** affording **37** and **38** improved the activity further for both compounds. Apart from **15**, **37** is the most active analog in the cytotoxicity assay with an IC_{50} of 1.4 μ M. The stability of the oximes in PBS buffer (pH 7.4) was tested and less than 5% hydrolysis to the parent ketones could be detected after 48 hours (data not shown).

The isogriseofulvin analogs tested in the multipolarity assay (**3** and **39-44**) were all inactive. In the cytotoxicity assay **35** retained the activity of **1**, while the rest were either inactive (**3**), showed low activity (**39, 44**) or were less potent (**40, 43** μM ; **41, 48** μM ; **42, 52** μM and **43, 27** μM).

Geodin (**56**) did not induce multipolar mitoses and was in that respect deemed inactive.³⁶ Geodin (**56**) is however twice as cytotoxic as griseofulvin when tested against the MDA-MB-231 cell line, with an IC_{50} of 9.9 μM opposed to 18 μM for **1**.

The reduced analogs **50** and **51** as well as the 3'-dimethyl analog **52** were inactive in both anti-cancer assays. In the 3' iodo series, **53** (22 μM) retained activity, the 2' propoxy analog (**54**, 8.0 μM) had increased activity and **55** exhibited low activity. **53** and **55** were inactive in the phenotype-based assay but **54** was more potent than **1**.

Anti-cancer SAR versus anti-fungal SAR. The difference between the anti-fungal and anti-cancer data is illustrated in Figure 4 demonstrating that most of the analogs had

increased potency against the cancer cell line but against the two fungal strains the activity was lower than for griseofulvin (**1**). Looking at the 4 position there were inconsistencies throughout all three cell types. The ethyl analog **5** was active against both fungi but inactive towards the cancer cells, while the bulkier naphthyl analog **8** was inactive against both fungi but more potent than griseofulvin against the MDA-MB-231 cells. Analogs **6** and **7** were more potent than **5** against *T. mentagrophytes*, but both compounds were inactive or showed low activity towards *T. rubrum*. The most potent analog towards the cancer cells (**15**) was inactive against both fungi and the isomer (**16**), which was amongst the most active compounds, only had low activity towards the two fungi. The two bulky analogs **25** and **27** as well as the dimer (**34**) inactive in the anti-fungal assays but were all more potent than griseofulvin against the cancer cell line. It is also worth noticing that geodin (**56**) was inactive against both fungi but twice as potent as **1** against the cancer cell line.

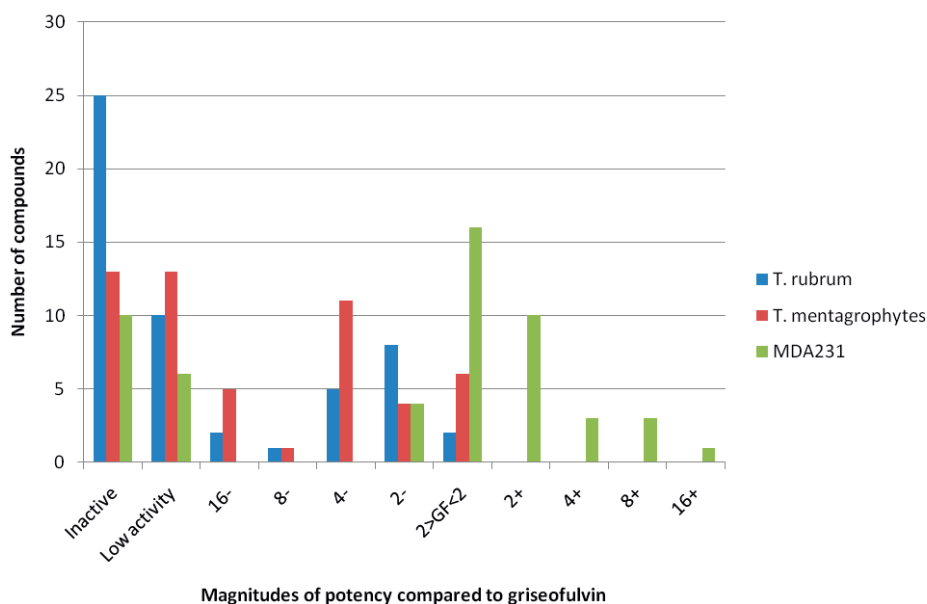


Figure 4. The IC_{50} values for each cell line have been normalized defining the activity of griseofulvin as having a value of 1. It is seen that of the 53 analogs the majority was less active against the two fungi opposed to the anti-cancer activity where most analogs were more active than griseofulvin.

The introduction of an oxime at the 4' position (**36**, **37** and **38**) decreased the potency compared to the parent compounds (**1**, **17** and **26**) against both fungi. The opposite effect was seen for the cancer cell line, where the introduction of this moiety increased the activity in all three compounds (**36**, **37** and **38**).

There were however some similar trends for all three cell types as well. A number of analogs were inactive or had low activity towards both fungi and cancer cells. Amongst these were the position 5 analogs (**9**, **10**), isogriseofulvin (**3**) and the series of isogriseofulvin analogs (**39-44**) with **35** as the sole exception. The two 6 phenols (**11**, **13**) were virtually inactive against all cell types, which was the same for **50-52**.

Conclusion

The first comparison of anti-fungal and anti-cancer SAR for griseofulvin analogs is presented in this work covering 53 analogs of griseofulvin as well as the natural product geodin (**56**). All compounds have been tested against *T. rubrum*, *T. mentagrophytes* and in a cytotoxicity assay against MDA-MB-231 breast cancer cells.

Even though there were similarities between the SAR of the two fungi, with some compounds showing no activity against both fungi and some active against both, there were also some differences. Analogs **46** and **48** retain the activity of griseofulvin against *T. mentagrophytes* but were two and four fold less active against *T. rubrum* than **1**, respectively. This is in accordance with reported observations by Crosse et al. that analogs differ in activity against different dermatophytes.¹¹

We show that there is a good correlation between the IC₅₀ values from analogs that were active in both the phenotype-based assay for spindle multipolarity²² and the cytotoxicity assay used in this work with an R² of 0.70. Comparing the two fungal SAR's with the anti-cancer cytotoxicity SAR afforded distinct differences. The analogs **8**, **25**, **27** and **38** were inactive or had low potency against both fungi, but either retained the activity (**8**) or were at least twofold more active against the cancer cells compared to **1**. The 2'-benzyloxy analog (**17**) was less active against both fungi in this study and against six dermatophytes in the study by Crosse et al.¹¹ This analog is however nine times more potent than **1** against the cancer cell line MDA-MB-231. It is noteworthy that the two dichloro compounds **15** and **16** were inactive against both fungi, but featured as some of the most cytotoxic agents against the cancer cell line.

The differences in activity observed for the two fungi and the MDA-MB-231 cell line could rise from a number of factors, such as transport over the cell membrane, efflux pumps or different modes of action. We find it unlikely that transport or efflux pumps could explain that the majority of analogs were more active against MDA-MB-231 cells and less active against both fungi. Our conclusion therefore is that the mode-of-action of griseofulvin(s) towards fungal and mammalian cells is different, making it unlikely that tubulin itself constitutes the main cellular target in both

fungi and mammalian cells. Since it has been shown that griseofulvin leads to mitotic arrest in both fungal and mammalian cells, an alternative explanation is that griseofulvin disrupts microtubule dynamics without directly interacting with tubulin, e.g. through interaction with microtubule-associated proteins (MAPs).

Experimental Section

The fungal micro broth dilution assay was performed in sterile flat bottomed microplates (cat.# 655101) and lids (cat.# 656161) from Greiner Bio-One GmbH (Frickenhausen, Germany). Each microplate accommodated five dilution series starting at 50 µM of a given analog. Each plate was also fitted with four negative and four positive control wells. The fungi *T. rubrum* (IBT29284) and *T. mentagrophytes* (IBT2724) are kept in the IBT fungal collection at department of Systems Biology at the Technical University of Denmark. The correct identity of the fungal cultures were verified by microscopy after the last test round in the antifungal assay. For testing against *T. rubrum* a fourfold dilution series was used, resulting in ten concentrations with the lowest at 0.2 µM. For *T. mentagrophytes* an eightfold dilution series was used resulting in 10 concentrations with the last one at 0.4 pM.

Inoculum preparation was performed in accordance with the document M38-A2³⁸ from the "Committee for Clinical Laboratory Standards". Inoculum was adjusted to 0.90-1.5 OD at 530 nm on a Genesys 10uv from Thermo Electron Corporation (Waltham, MA, US). The inoculums were diluted fiftyfold with RPMI media (with L-glutamine, without NaHCO₃, cat.# R6504 in MOPS buffer (cat.# 69947)) both from Sigma-Aldrich (St Louis, MO, US). The pH was adjusted to 7.00 with 1 M NaOH and the volume with MilliQ water from a MilliQ gradient fitted with a Millipore 0.22 µm filter (cat.# MPGL04001) both from Millipore (Billerica, MA, US).

Each well contained 99µL RPMI media with inoculum, 99µL MilliQ water and 2 µL DMSO. Microplates were incubated for 7 days at 37 °C in a WTB binder incubator from VWR (Radnor, PA, US) at which point the OD of the wells were measured at 530 µM on a BioTek® Synergy 2 (Brøndby, Denmark) using Gen5TM 1.10.8 software. The half maximal inhibitory concentration (IC₅₀) values were obtained with GraphPad Prism5 (ver. 5.04) from GraphPad Software (La Jolla, CA, US) using the "nonlinear regression, sigmoidal dose-response (variable slope)" function. The fitting method used was "automatic outlier elimination",³⁹ which eliminated less than 1.1% data points.

¹H NMR spectra were recorded using either a Varian Unity Inova 500 MHz spectrometer or a Varian Mercury 300 MHz spectrometer both from Agilent (Santa Clara, CA, US). ¹³C NMR spectra were recorded using either a Varian Mercury 300 MHz or a Bruker AC 200 MHz from Bruker Optics (Ettlingen, Germany). Chemical shifts were measured in ppm and coupling constants in Hz. When CDCl₃ was used

as solvent the residual peak was used as internal reference at δ 7.27 for ^1H NMR and δ 77.00 for ^{13}C NMR spectra.

IR spectra were recorded using a Bruker Alpha ATR and measured in cm^{-1} . All melting points are uncorrected. TLC was performed on aluminium sheets precoated with silica gel 60 F254 (Merck 1.05554.0001).

High-resolution LC-DAD-MS was performed on an Agilent 1100 system equipped with a photodiode array detector (DAD) and coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass, Manchester, UK) with a Z-spray electrospray ionisation (ESI) source and a LockSpray probe ($\text{M}+\text{H}$ 556.2771) and controlled by MassLynx 4.0 software. LC-MS calibration from m/z 100–900 was done with a PEG mixture. Standard separation involved a LUNA 2 column with an acetonitrile (50 ppm TFA) in water gradient starting from 15% to 100% over 25 minutes with a flow rate of 0.3 mL/min.

Compounds were visualized by charring after dipping in a solution of 1% KMnO_4 , 6.7% K_2CO_3 and 0.08% NaOH in water. UV visualization was done using a Model UVGL-25 Mineralight Lamp.

Microanalyses were obtained from H. Kolbe Mikroanalytisches Laboratorium in Mülheim an der Ruhr, Germany.

General Procedure for the Synthesis of position 4 ethers (7 and 8)

The appropriate alkyl bromide (1.2 mmol, 3 equiv.) was added to a solution of **4** (0.4 mmol, 1 equiv), Ag_2O (1.2 mmol, 3 equiv), and 1,4-dioxane (5 mL). The mixture was stirred at 50 °C for 18 h and then cooled to 20 °C. EtOAc (10 mL) was added to the solution and the mixture was washed with brine (15 mL). The aqueous phase was extracted with EtOAc (2×10 mL), the combined org. phases were dried (MgSO_4), and concentrated. The residue was purified by column chromatography (EtOAc:heptane 1:3) to afford the desired product.

(2*S*,6'*R*)-(7-Chloro-6-methoxy-4-pentoxo-benzofuran-3-one)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-ene-4'-one) **7**

Yield: 80 mg (44 %) (yellow oil); R_f -value (EtOAc/heptane, 2:1): 0.39; IR (neat, cm^{-1}): 1709, 1664, 1612, 1586; ^1H NMR (CDCl_3 , 500 MHz): δ 6.11 (1H, s), 5.52 (1H, s), 4.13–4.05 (2H, m), 4.00 (3H, s), 3.61 (3H, s), 3.04 (1H, dd, $J = 16.7$, 13.5 Hz), 2.82 (1H, d, $J = 4.7$, 6.7, 13.5 Hz), 2.41 (1H, dd, $J = 16.7$, 4.7 Hz), 1.92–1.85 (2H, m), 1.50–1.42 (2H, m), 1.42–1.34 (2H, m), 0.95 (3H, d, $J = 6.7$ Hz), 0.92 (3H, t, $J = 7.2$ Hz); ^{13}C NMR (CDCl_3 , 50 MHz): δ 196.9, 192.0, 170.9, 169.4, 164.4, 157.4, 105.2, 104.7, 96.9, 90.6, 90.3, 69.6, 56.9, 56.6, 40.0, 36.5, 28.4, 27.9, 22.3 14.2, 13.9; $[\alpha]_D^{20} = +270^\circ$ ($c = 0.3$ in CHCl_3); HRMS (ESI⁺) calcd for $[\text{M}+\text{H}]^+$ $[\text{C}_{21}\text{H}_{36}\text{ClO}_6]^+$ 409.1418, found 409.1419.

(2*S*,6'*R*)-(7-Chloro-6-methoxy-4-(naphthalen-1-ylmethoxy)-benzofuran-3-one)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-ene-4'-one) **8**

Yield: 25 mg (12 %) (yellow oil); R_f -value (EtOAc/heptane, 2:1): 0.35; IR (neat, cm^{-1}): 1704, 1662, 1612, 1588; ^1H NMR

(CDCl_3 , 500 MHz): δ 7.94–7.82 (4H, m), 7.57 (1H, dd, $J = 8.5$, 0.9 Hz), 7.52–7.48 (2H, m), 6.23 (1H, s), 5.56 (1H, s), 5.44 (2H, s), 3.92 (3H, s), 3.63 (3H, s), 3.07 (1H, dd, $J = 16.7$, 13.5 Hz), 2.86 (1H, ddq, $J = 13.5$, 6.7, 4.7 Hz), 2.45 (1H, dd, $J = 16.7$, 4.7 Hz), 0.99 (3H, d, $J = 6.7$ Hz); ^{13}C NMR (CDCl_3 , 50 MHz): δ 197.0, 192.2, 170.8, 169.5, 164.3, 156.7, 133.2, 132.9, 128.8, 128.0, 127.8, 126.5, 126.4 (2C), 126.1, 124.5, 105.7, 104.9, 97.6, 91.8, 90.7, 71.5, 56.9, 56.7, 40.1, 36.5, 14.3; $[\alpha]_D^{20} = +123^\circ$ ($c = 0.6$ in CHCl_3); HRMS (ESI⁺) calcd for $[\text{M}+\text{H}]^+$ $[\text{C}_{27}\text{H}_{34}\text{ClO}_6]^+$ 479.1261, found 479.1266.

(2*S*,6'*R*)-(7-Chloro-6-ethoxy-4-methoxy-benzofuran-3-one)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-ene-4'-one) **14**

Ethyl bromide (0.9 mmol, 3 equiv) was added to a solution of **12** (0.3 mmol, 1 equiv), Ag_2O (0.9 mmol, 3 equiv), and 1,4-dioxane (3 mL). The mixture was stirred at 50 °C for 18 h and then cooled to 20 °C. EtOAc (10 mL) was added to the solution and the mixture was washed with brine (15 mL). The aqueous phase was extracted with EtOAc (2×10 mL), the combined org. phases were dried (MgSO_4), and concentrated. The following process was repeated three times: The residue was dissolved in MeOH (10 mL) and CSA (0.1 mmol) was added, the mixture was heated to 65 °C for 18 h. EtOAc (10 mL) was added to the solution and the mixture was washed with sat. aq. NaHCO_3 (15 mL). The aqueous phase was extracted with EtOAc (2×10 mL), the combined org. phases were dried (MgSO_4), and concentrated. The residue was purified by column chromatography (EtOAc:heptane 1:3) to afford the desired product **14**.

Yield: 5 mg (5 %) (yellow oil); R_f -value (EtOAc/heptane, 5:1): 0.43; IR (neat, cm^{-1}): 1709, 1666, 1613, 1587 (Litt.⁴⁰); ^1H NMR (CDCl_3 , 300 MHz): δ 6.13 (1H, s), 5.56 (1H, s), 4.25 (2H, q, $J = 7.0$ Hz), 3.98 (3H, s), 3.63 (3H, s), 3.05 (1H, dd, $J = 16.7$, 13.4 Hz), 2.86 (1H,), 2.45 (1H, dd, $J = 16.7$, 4.7 Hz), 1.56 (3H, t, $J = 7.0$ Hz), 0.98 (3H, d, $J = 6.7$ Hz); ^{13}C NMR (CDCl_3 , 50 MHz): δ 197.0, 192.4, 170.8, 169.6, 164.1, 157.7, 104.9 (2C), 93.4, 90.7, 90.2, 65.8, 56.6, 56.3, 40.1, 36.4, 14.6, 14.2; $[\alpha]_D^{20} = +252^\circ$ ($c = 0.5$ in CCl_4) (Litt.²⁷); HRMS (ESI⁺) calcd for $[\text{M}+\text{H}]^+$ $[\text{C}_{18}\text{H}_{20}\text{ClNO}_6]^+$ 367.0948, found 367.0949.

General Procedure for the synthesis of 2' enol ethers (19, 21, 22, 26, 28–33)

To a solution of **16** (0.65 mmol, 1 equiv) in 1,4-dioxane (3 mL, 0.2 M) was added the desired alcohol (1.30 mmol, 2 equiv) and DBU (1.63 mmol, 2.5 equiv). The mixture was heated to 100 °C and stirred for 12 h. The mixture was then cooled to 20 °C, and excess reagent was quenched with sat. aq. NH_4Cl (30 mL). The aqueous phase was extracted with EtOAc (3×30 mL) and the combined organic phases were dried (MgSO_4) and concentrated. The residue was purified by column chromatography (EtOAc:heptane: 1:3) affording the product. When possible the product was recrystallized from EtOAc/heptane.

(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-(4-fluorobenzoyloxy)-6'-methyl-cyclohex-2'-ene-4'-one) 19

Yield: 116 mg (46 %) (white crystals); R_f -value (EtOAc/heptane, 5:1): 0.46; m.p. 103-105 °C; IR (neat, cm^{-1}): 1704, 1661, 1610, 1587; ^1H NMR (CDCl_3 , 300 MHz): δ 7.19-7.11 (2H, m), 7.02-6.93 (2H, m), 6.10 (1H, s), 5.58 (1H, s), 4.87 (1H, d, $J = 12.1$ Hz), 4.76 (1H, d, $J = 12.1$ Hz), 4.02 (3H, s), 3.96 (3H, s), 3.05 (1H, dd, $J = 16.5$, 13.4 Hz), 2.93-2.79 (1H, m), 2.43 (1H, dd, $J = 16.5$, 4.5 Hz), 0.98 (3H, d, $J = 6.7$ Hz); ^{19}F NMR (CDCl_3) δ -114.29 (m); ^{13}C NMR (CDCl_3 , 50 MHz): δ 196.7, 192.2, 169.3, 164.8, 164.5, 159.9, 157.6, 130.3, 128.6, 128.4, 115.6, 115.2, 105.8, 105.0, 97.0, 90.6, 89.5, 69.9, 56.9, 56.3, 39.9, 36.3, 14.1; $[\alpha]_D^{20} = +118^\circ$ (c = 1 in CHCl_3); HRMS (ESI $^+$) calcd for $[\text{M}+\text{H}]^+$ $[\text{C}_{23}\text{H}_{21}\text{ClF}_2\text{O}_6]^+$ 447.1011, found 447.1009.

(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-(4-trifluoromethylbenzyloxy)-6'-methyl-cyclohex-2'-ene-4'-one) 21

Yield: 128 mg (46 %) (white crystals); R_f -value (EtOAc/heptane, 5:1): 0.58; m.p. 103-105 °C; IR (neat, cm^{-1}): 1705, 1664, 1612, 1589; ^1H NMR (CDCl_3 , 300 MHz): δ 7.56 (2H, d, $J = 8.2$ Hz), 7.29 (2H, d, $J = 8.2$ Hz), 6.12 (1H, s), 5.58 (1H, s), 4.97 (1H, d, $J = 12.9$ Hz), 4.85 (1H, d, $J = 12.9$ Hz), 4.04 (3H, s), 3.97 (3H, s), 3.07 (1H, dd, $J = 16.5$, 13.4 Hz), 2.97-2.77 (1H, m), 2.45 (1H, dd, $J = 16.5$, 4.5 Hz), 1.00 (3H, d, $J = 6.6$ Hz); ^{19}F NMR (CDCl_3) δ -63.30 (s); ^{13}C NMR (CDCl_3 , 50 MHz): δ 196.7, 192.2, 169.5, 169.1 (2C), 164.7, 157.8, 138.6, 130.3 (q, $J = 32.4$ Hz), 126.6 (2C), 125.5, 106.1, 105.1, 97.2, 90.7, 89.6, 69.6, 57.0, 56.4, 40.0, 36.4, 14.2; $[\alpha]_D^{20} = +81^\circ$ (c = 1 in CHCl_3); HRMS (ESI $^+$) calcd for $[\text{M}+\text{H}]^+$ $[\text{C}_{24}\text{H}_{21}\text{ClF}_3\text{O}_6]^+$ 497.0979, found 497.0974.

(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-(4-(hydroxymethyl)-benzoyloxy)-6'-methyl-cyclohex-2'-ene-4'-one) 22

Yield: 120 mg (47 %) (white crystals); R_f -value ($\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{HOAc}$, 89:10:1): 0.48; m.p. 160-163 °C; IR (neat, cm^{-1}): 1704, 1661, 1613, 1589; ^1H NMR (CDCl_3 , 500 MHz): δ 7.30 (2H, d, $J = 8.0$ Hz), 7.17 (2H, d, $J = 8.0$ Hz), 6.11 (1H, s), 5.59 (1H, s), 4.91 (1H, d, $J = 12.3$ Hz), 4.81 (1H, d, $J = 12.3$ Hz), 4.67 (2H, s), 4.02 (3H, s), 3.96 (3H, s), 3.05 (1H, dd, $J = 16.7$, 13.4 Hz), 2.86 (1H, ddq, $J = 13.4$, 6.7, 4.7 Hz), 2.43 (1H, dd, $J = 16.7$, 4.7 Hz), 0.99 (3H, d, $J = 6.7$ Hz); ^{13}C NMR (CDCl_3 , 50 MHz): δ 197.1, 192.4, 169.5 (2C), 164.5, 157.7, 140.9, 133.9, 127.1 (2C), 126.9 (2C), 105.9, 105.1, 97.1, 90.7, 89.5, 70.5, 64.9, 57.0, 56.3, 40.0, 36.4, 14.3; $[\alpha]_D^{20} = +148^\circ$ (c = 0.6 in CHCl_3); HRMS (ESI $^+$) calcd for $[\text{M}+\text{H}]^+$ $[\text{C}_{24}\text{H}_{24}\text{ClO}_7]^+$ 459.1211, found 459.1212.

(1'S,6'R)-(7-Chloro-4,6-dimethoxy-6'-methyl-2'-(naphthalen-1-ylmethoxy)-spiro[benzofuran-2,1'-cyclohex[2]ene]-3,4'-dione 26

Yield: 354 mg (53 %) (white crystals); R_f -value (EtOAc/heptane, 5:1): 0.46; m.p. 180-182 °C; IR (neat, cm^{-1}): 1706, 1662, 1613, 1589; ^1H NMR (500 MHz, CDCl_3): δ

7.84-7.76 (3H, m), 7.51-7.44 (2H, m), 7.39-7.33 (2H, m), 6.00 (1H, s), 5.77 (1H, s), 5.31 (1H, d, $J = 19.6$ Hz), 5.28 (1H, d, $J = 19.6$ Hz), 3.96 (3H, s), 3.93 (3H, s), 3.08 (1H, dd, $J = 16.7$, 13.5 Hz), 2.88 (1H, ddq, $J = 13.5$, 6.7, 4.6 Hz), 2.45 (1H, dd, $J = 16.7$, 4.6 Hz), 0.98 (3H, d, $J = 6.7$ Hz); ^{13}C -NMR (75 MHz, CDCl_3): δ 197.0, 192.4, 169.6, 169.4, 164.4, 157.6, 133.4, 131.0, 129.7, 129.3, 128.5, 126.6, 126.3, 125.8, 124.9, 123.2, 105.9, 105.0, 97.0, 90.7, 89.3, 70.0, 56.8, 56.2, 40.0, 36.2, 14.2; $[\alpha]_D^{20} = +112$ (c 1.0, CHCl_3); HRMS (ESI $^+$) calcd for $[\text{M}+\text{H}]^+$ $[\text{C}_{27}\text{H}_{24}\text{ClO}_6]^+$ 479.1261, found 479.1258.

(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-(pyridin-2-ylmethoxy)-6'-methyl-cyclohex-2'-ene-4'-one) 28

Yield: 118 mg (49 %) (white crystals); R_f -value ($\text{MeOH}/\text{CH}_2\text{Cl}_2$, 1:9): 0.70; m.p. 222-224 °C; IR (neat, cm^{-1}): 1697, 1661, 1612, 1586; ^1H NMR (CDCl_3 , 300 MHz): δ 8.50 (1H, ddd, $J = 4.8$, 1.7, 1.0 Hz), 7.63 (1H, ddd, $J = 7.9$, 7.7, 1.7 Hz), 7.18 (1H, ddd, $J = 7.7$, 4.8, 1.0 Hz), 7.14 (1H, ddd, $J = 7.9$, 1.0, 1.0 Hz), 6.14 (1H, s), 5.60 (1H, s), 5.05 (1H, d, $J = 13.9$ Hz), 4.94 (1H, d, $J = 13.9$ Hz), 4.04 (3H, s), 3.98 (3H, s), 3.06 (1H, dd, $J = 16.4$, 13.4 Hz), 2.97-2.78 (1H, m), 2.44 (1H, dd, $J = 16.4$, 4.5 Hz), 1.00 (3H, d, $J = 6.6$ Hz); ^{13}C NMR (CDCl_3 , 50 MHz): δ 196.7, 192.1, 169.5, 169.0, 164.6, 157.8, 154.8, 149.0, 136.9, 122.8, 120.3, 106.2, 105.1, 97.1, 90.7, 89.6, 70.9, 57.0, 56.3, 40.0, 36.3, 14.2; $[\alpha]_D^{20} = +163^\circ$ (c = 1 in CHCl_3); HRMS (ESI $^+$) calcd for $[\text{M}+\text{H}]^+$ $[\text{C}_{22}\text{H}_{21}\text{ClNO}_6]^+$ 430.1057, found 430.1059.

(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-(pyridin-3-ylmethoxy)-6'-methyl-cyclohex-2'-ene-4'-one) 29

Yield: 76 mg (32 %) (yellow oil); R_f -value ($\text{MeOH}/\text{CH}_2\text{Cl}_2$, 1:9): 0.65; IR (neat, cm^{-1}): 1705, 1663, 1613, 1589; ^1H NMR (CDCl_3 , 300 MHz): δ 8.55 (1H, dd, $J = 4.8$, 1.1 Hz), 8.45 (1H, d, $J = 1.1$ Hz), 7.54 (1H, d, $J = 7.8$ Hz), 7.27 (1H, dd, $J = 7.8$, 4.8 Hz), 6.13 (1H, s), 5.64 (1H, s), 4.95 (1H, d, $J = 12.32$ Hz), 4.85 (1H, d, $J = 12.31$ Hz), 4.04 (3H, s), 3.99 (3H, s), 3.09 (1H, dd, $J = 16.4$, 13.5 Hz), 2.90 (1H, ddq, $J = 13.5$, 6.6, 4.4 Hz), 2.47 (1H, dd, $J = 16.4$, 4.4 Hz), 1.01 (3H, d, $J = 6.6$ Hz); ^{13}C NMR (CDCl_3 , 75 MHz): δ 196.8, 192.2, 169.4, 169.2, 164.6, 157.7, 149.7, 148.2, 134.6, 130.2, 123.5, 106.0, 105.0, 97.1, 90.6, 89.5, 68.3, 57.0, 56.3, 40.0, 36.4, 14.2; $[\alpha]_D^{20} = +181^\circ$ (c = 0.3 in CCl_3); HRMS (ESI $^+$) calcd for $[\text{M}+\text{H}]^+$ $[\text{C}_{22}\text{H}_{21}\text{ClNO}_6]^+$ 430.1057, found 430.1054.

(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-(pyridin-4-ylmethoxy)-6'-methyl-cyclohex-2'-ene-4'-one) 30

Yield: 70 mg (29 %) (yellow oil); R_f -value ($\text{MeOH}/\text{CH}_2\text{Cl}_2$, 1:9): 0.60; IR (neat, cm^{-1}): 1706, 1664, 1615, 1590; ^1H NMR (CDCl_3 , 300 MHz): δ 8.53 (2H, d, $J = 4.8$ Hz), 7.08 (2H, d, $J = 4.8$ Hz), 6.13 (1H, s), 5.55 (1H, s), 4.92 (1H, d, $J = 13.5$ Hz), 4.79 (1H, d, $J = 13.5$ Hz), 4.04 (3H, s), 3.97 (3H, s), 3.07 (1H, dd, $J = 16.4$, 13.5 Hz), 2.89 (1H, ddq, $J = 13.5$, 4.2, 6.6 Hz), 2.46 (1H, dd, $J = 16.4$, 4.2 Hz), 1.01 (3H, d, $J = 6.6$ Hz); ^{13}C NMR (CDCl_3 , 50 MHz): δ 196.6, 192.1, 169.5,

168.9, 164.7, 157.8, 150.0 (2C), 143.6, 120.7 (2C), 106.1, 105.1, 97.1, 90.6, 89.6, 68.5, 57.0, 56.4, 40.0, 36.3, 14.2; $[\alpha]_D^{20} = +148^\circ$ ($c = 0.3$ in CCl_3); HRMS (ESI⁺) calcd for $[\text{M}+\text{H}]^+$ $[\text{C}_{22}\text{H}_{21}\text{ClNO}_6]^+$ 430.1057, found 430.1053.

(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-2-methoxybenzyloxy-6'-methyl-cyclohex-2'-ene-4'-one) 31

Yield: 82 mg (43 %) (white crystals); R_f -value (EtOAc/heptane, 5:1): 0.43; m.p. 92-95 °C; IR (neat, cm^{-1}): 1705, 1660, 1610, 1588; ^1H NMR (CDCl_3 , 500 MHz): δ 7.23 (1H, m), 7.09 (1H, m), 6.86 (1H, m), 6.81 (1H, m), 6.11 (1H, s), 5.64 (1H, s), 4.95 (1H, d, $J = 13.2$ Hz), 4.88 (1H, d, $J = 13.2$ Hz), 4.03 (3H, s), 3.96 (3H, s), 3.78 (3H, s), 3.06 (1H, dd, $J = 16.7, 13.5$ Hz), 2.87 (1H, ddq, $J = 13.5, 4.7, 6.6$ Hz), 2.43 (1H, dd, $J = 16.7, 4.7$ Hz), 0.99 (3H, d, $J = 6.6$ Hz); ^{13}C NMR (CDCl_3 , 50 MHz): δ 197.0, 192.3, 169.6, 169.5, 164.4, 157.6, 156.3, 129.0, 127.3, 122.9, 120.4, 110.0, 105.6, 105.1, 97.1, 90.7, 89.4, 66.2, 56.9, 56.2, 55.2, 39.9, 36.2, 14.2; $[\alpha]_D^{20} = +124^\circ$ ($c = 1$ in CHCl_3); HRMS (ESI⁺) calcd for $[\text{M}+\text{H}]^+$ $[\text{C}_{24}\text{H}_{24}\text{ClO}_7]^+$ 459.1211, found 459.1216.

(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-3-methoxybenzyloxy-6'-methyl-cyclohex-2'-ene-4'-one) 32

Yield: 101 mg (52 %) (white crystals); R_f -value (EtOAc/heptane, 5:1): 0.56; m.p. 166-167 °C; IR (neat, cm^{-1}): 1707, 1662, 1613, 1589; ^1H NMR (CDCl_3 , 300 MHz): δ 7.23-7.15 (1H, m), 6.82-6.68 (3H, m), 6.11 (1H, s), 5.59 (1H, s), 4.91 (1H, d, $J = 12.5$ Hz), 4.79 (1H, d, $J = 12.5$ Hz), 4.02 (3H, s), 3.96 (3H, s), 3.73 (3H, s), 3.06 (1H, dd, $J = 16.4, 13.4$ Hz), 2.96-2.77 (1H, m), 2.44 (1H, dd, $J = 16.4, 4.5$ Hz), 0.99 (3H, d, $J = 6.6$ Hz); ^{13}C NMR (CDCl_3 , 50 MHz): δ 196.9, 192.4, 169.6, 169.4, 164.5, 159.8, 157.7, 136.1, 129.6, 118.6, 114.6, 110.8, 106.0, 105.2, 97.1, 90.8, 89.5, 70.4, 57.0, 56.3, 55.1, 40.0, 36.3, 14.3; $[\alpha]_D^{20} = +151^\circ$ ($c = 1$ in CHCl_3); HRMS (ESI⁺) calcd for $[\text{M}+\text{H}]^+$ $[\text{C}_{24}\text{H}_{24}\text{ClO}_7]^+$ 459.1211, found 459.1217.

(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-4-methoxybenzyloxy-6'-methyl-cyclohex-2'-ene-4'-one) 33

Yield: 62 mg (51 %) (white crystals); R_f -value (EtOAc/heptane, 5:1): 0.46; m.p. 148-150 °C; IR (neat, cm^{-1}): 1705, 1660, 1611, 1588; ^1H NMR (CDCl_3 , 300 MHz): δ 7.12 (2H, d, $J = 8.7$ Hz), 6.82 (2H, d, $J = 8.7$ Hz), 6.09 (1H, s), 5.60 (1H, s), 4.85 (1H, d, $J = 11.8$ Hz), 4.74 (1H, d, $J = 11.8$ Hz), 4.02 (3H, s), 3.96 (3H, s), 3.78 (3H, s), 3.05 (1H, dd, $J = 16.5, 13.3$ Hz), 2.94-2.76 (1H, m), 2.43 (1H, dd, $J = 16.5, 4.5$ Hz), 0.98 (3H, d, $J = 6.6$ Hz); ^{13}C NMR (CDCl_3 , 50 MHz): δ 197.0, 192.4, 169.6 (2C), 164.5, 159.5, 157.7, 128.5 (2C), 126.6, 113.9 (2C), 105.9, 105.2, 97.2, 90.8, 89.5, 70.7, 56.9, 56.3, 55.2, 40.0, 36.4, 14.2; $[\alpha]_D^{20} = +103^\circ$ ($c = 1$ in CHCl_3); HRMS (ESI⁺) calcd for $[\text{M}+\text{H}]^+$ $[\text{C}_{24}\text{H}_{24}\text{ClO}_7]^+$ 459.1211, found 459.1214.

Griseofulvin dimer 34

Yield: 20 mg (5 %) (yellow oil); R_f -value ($\text{CH}_2\text{Cl}_2:\text{MeOH}:\text{HOAc}$, 89:10:1): 0.63; IR (neat, cm^{-1}):

1705, 1662, 1613, 1588; ^1H NMR (CDCl_3 , 300 MHz): δ 7.10 (4H, s), 6.11 (2H, s), 5.56 (2H, s), 4.88 (2H, d, $J = 12.4$ Hz), 4.76 (2H, d, $J = 12.4$ Hz), 4.03 (6H, s), 3.96 (6H, s), 3.04 (2H, dd, $J = 16.7, 13.4$ Hz), 2.85 (2H, ddq, $J = 13.4, 4.7, 6.7$ Hz), 2.43 (2H, dd, $J = 16.7, 4.7$ Hz), 0.99 (6H, d, $J = 6.7$ Hz); ^{13}C NMR (CDCl_3 , 50 MHz): δ 196.9 (2C), 192.4 (2C), 169.5 (4C), 164.6 (2C), 157.7 (2C), 134.7 (2C), 126.7 (4C), 105.9 (2C), 105.2 (2C), 97.2 (2C), 90.8 (2C), 89.6 (2C), 70.2 (2C), 57.0 (2C), 56.4 (2C), 40.0 (2C), 36.4 (2C), 14.3 (2C); $[\alpha]_D^{20} = +167^\circ$ ($c = 0.3$ in CHCl_3); HRMS (ESI⁺) calcd for $[\text{M}+\text{H}]^+$ $[\text{C}_{40}\text{H}_{37}\text{Cl}_2\text{O}_{12}]^+$ 779.1662, found 779.1658.

(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-(naphthalen-1-ylmethoxy)-6'-methylcyclohex-2'-ene-4'-one-4'-oxime) 38

To a solution of **26** (0.08 mmol, 1.0 equiv.) in EtOH (2 mL) and DMSO (1 mL) was added hydroxylamine hydrochloride (0.30 mmol, 3.5 equiv.) and sodium acetate (0.37 mmol, 4.3 equiv.). The mixture was stirred at 75 °C for 18 hours, allowed to reach 20 °C and diluted with EtOAc (5 mL). The mixture was washed with brine (3×4 mL). The organic phase was dried (MgSO_4) and concentrated. The crude mixture was purified by column chromatography (EtOAc:heptane 1:1) to afford **38** (40 mg, 96 %) as a yellow oil. R_f -values (EtOAc/heptane, 5:1): 0.60 and 0.51; IR (neat, cm^{-1}): 1704, 1613, 1590; ^1H NMR (500 MHz, CDCl_3): δ 7.82-7.73 (3H, m), 7.50-7.42 (2H, m), 7.37-7.32 (2H, m), 6.54 (0.4H, s), 5.99 (0.6H, s), 5.96 (0.4H, s), 5.84 (0.6H, s), 5.31 (0.4H, d, $J = 12.0$ Hz), 5.29 (0.6H, d, $J = 12.0$ Hz), 5.26 (0.4H, d, $J = 11.9$ Hz), 5.23 (0.6H, d, $J = 11.9$ Hz), 3.95 (1.8H, s), 3.93 (1.2H, s), 3.91 (1.8H, s), 3.90 (1.2H, s), 3.15 (0.6 H, dd, $J = 16.8, 4.9$ Hz), 3.06 (0.4 H, dd, $J = 15.0, 13.3$ Hz), 2.75 (0.6 H, dd, $J = 16.8, 13.1$ Hz), 2.70-2.57 (1 H, m), 2.44 (0.4 H, dd, $J = 15.0, 4.1$ Hz), 0.98 (1.8H, d, $J = 6.7$ Hz), 0.98 (1.2H, d, 6.8 Hz); ^{13}C NMR (75 MHz, CDCl_3): δ 194.0 (0.5C, s), 193.9 (0.5C, s), 169.3 (0.5C, s), 164.0 (0.5C, s), 160.0 (0.5C, s), 157.3 (0.5C, s), 157.3 (0.5C, s), 157.3 (0.5C, s), 155.0 (0.5C, s), 151.8 (0.5C, s), 133.3 (0.5C, s), 131.2 (0.5C, s), 131.1 (0.5C, s), 130.8 (0.5C, s), 130.6 (0.5C, s), 128.9 (0.5C, s), 128.9 (0.5C, s), 128.8 (0.5C, s), 128.3 (0.5C, s), 128.2 (0.5C, s), 128.1 (0.5C, s), 126.3 (0.5C, s), 126.3 (0.5C, s), 126.2 (0.5C, s), 126.0 (0.5C, s), 125.7 (0.5C, s), 125.6 (0.5C, s), 125.2 (0.5C, s), 125.0 (0.5C, s), 124.9 (0.5C, s), 123.6 (0.5C, s), 123.5 (0.5C, s), 105.6 (0.5C, s), 105.5 (0.5C, s), 100.5 (0.5C, s), 96.9 (0.5C, s), 96.8 (0.5C, s), 93.9 (0.5C, s), 91.5 (0.5C, s), 91.4 (0.5C, s), 89.0 (0.5C, s), 89.0 (0.5C, s), 69.4 (0.5C, s), 69.0 (0.5C, s), 56.7 (1 C, s), 56.1 (0.5C, s), 56.1 (0.5C, s), 36.4 (0.5C, s), 35.2 (0.5C, s), 30.9 (0.5C, s), 25.5 (0.5C, s), 14.4 (0.5 C, s), 14.3 (0.5 C, s); $[\alpha]_D^{20} = +112^\circ$ ($c = 1.0$ in CDCl_3); HRMS (ESI⁺) calcd for $\text{M}+\text{H}$ $[\text{C}_{27}\text{H}_{25}\text{ClNO}_6]^+$ 494.1370, found 494.1371.

The analog **35** was synthesized according to the general procedure for 2' enol ethers with the starting material being **15** instead of **16**.

(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(4'-(3-methoxybenzyl)oxy-6'-methyl-cyclohex-3'-en-2'-one) 35

Yield: 83 mg (32 %) (white crystals); R_f -value (EtOAc/heptane, 5:1): 0.65; m.p. 173-175 °C; IR (neat, cm^{-1}): 1691, 1653, 1605, 1584; ^1H NMR (CDCl_3 , 300 MHz): δ 7.37-7.27 (1H, m), 7.02-6.84 (3H, m), 6.09 (1H, s), 5.54 (1H, d, $J = 1.0$ Hz), 5.01-4.85 (2H, m), 4.01 (3H, s), 3.93 (3H, s), 3.84 (3H, s), 3.26 (1H, ddd, $J = 17.5$, 11.9, 1.0 Hz), 2.96-2.80 (1H, m), 2.54 (1H, dd, $J = 17.5$, 5.6 Hz), 1.03 (3H, d, $J = 6.6$ Hz); ^{13}C NMR (CDCl_3 , 50 MHz): δ 191.7, 188.8, 177.7, 169.7, 164.5, 159.9, 157.7, 136.0, 129.7, 120.0, 114.2, 113.2, 105.1, 100.7, 97.3, 95.1, 89.5, 71.0, 57.0, 56.2, 55.3, 35.2, 33.2 14.5; $[\alpha]_D^{20} = +179^\circ$ ($c = 0.6$ in CCl_3); HRMS (ESI $^+$) calcd for $[\text{M}+\text{H}]^+$ $[\text{C}_{24}\text{H}_{24}\text{ClO}_7]^+$ 459.1211, found 459.1216.

General procedure for the synthesis of enol ethers by solvolysis (42 and 43).

CSA (0.1 mmol, 0.1 equiv.) was added to a solution of griseofulvic acid (0.6 mmol, 1 equiv.) in the appropriate alcohol (6 mL, 0.1 M). The mixture was stirred at 100 °C for 6 hours, and then cooled to 20 °C. EtOAc (20 mL) was added to the solution and the mixture was washed with sat. aq. NaH_2PO_4 (20 mL) and water (20 mL). The combined aqueous phases were extracted with EtOAc (3×20 mL), the combined org. phases were dried (MgSO_4) and concentrated. The residue was purified by column chromatography (toluene: CH_2Cl_2 :EtOAc 7:7:1) to afford the desired products. When possible the products were recrystallized from EtOAc/Heptane.

(2S,6'R)-(7-chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(4'-cyclopropylmethoxy-6'-methyl-cyclohex-3'-en-2'-one) 42

Yield: 520 mg (21 %) (white crystals); R_f -value (EtOAc/heptane, 5:1): 0.61; m.p. 160-162 °C; IR (neat, cm^{-1}): 1703, 1658, 1610, 1592; ^1H NMR (CDCl_3 , 500 MHz): δ 6.06 (1H, s), 5.36 (1H, d, $J = 1.2$ Hz), 3.99 (3H, s), 3.89 (3H, s), 3.73 (2H, dq, $J = 10.4$, 7.2 Hz), 3.20 (1H, dd, $J = 17.5$, 12.2 Hz), 2.88-2.79 (1H, m), 2.49 (1H, dd, $J = 17.5$, 5.9 Hz), 1.27-1.17 (1H, m), 1.01 (3H, d, $J = 6.6$ Hz), 0.67-0.60 (2H, m), 0.35-0.29 (2H, m); ^{13}C NMR (CDCl_3 , 50 MHz): δ 191.7, 188.7, 178.1, 169.6, 164.4, 157.6, 105.1, 99.9, 97.2, 95.0, 89.4, 74.0, 56.9, 56.2, 35.1, 33.2, 14.4, 9.3, 3.2 (2C); $[\alpha]_D^{20} = +180$ ($c = 0.4$ in CHCl_3); HRMS (ESI $^+$) calcd for $[\text{M}+\text{H}]^+$ $[\text{C}_{20}\text{H}_{22}\text{ClO}_6]^+$ 393.1105, found 393.1114.

(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(4'-cyclopentoxymethoxy-6'-methyl-cyclohex-3'-en-2'-one) 43

Yield: 448 mg (36 %) (white crystals); R_f -value (EtOAc/heptane, 5:1): 0.61; m.p. 200-202 °C; IR (neat, cm^{-1}): 1696, 1648, 1614, 1585; ^1H NMR (CDCl_3 , 300 MHz): δ 6.03 (1H, s), 5.35 (1H, s), 4.62-4.67 (1H, m), 3.96 (3H, s), 3.82 (3H, s), 3.08 (1H, dd, $J = 17.6$, 12.1 Hz), 2.82-2.73 (1H, m), 2.37 (1H, dd, $J = 17.6$, 5.7 Hz), 1.89-1.52 (8H, m), 0.96 (3H, d, $J = 6.7$ Hz); ^{13}C NMR (CDCl_3 , 50 MHz): δ 191.6, 188.7, 177.3, 169.5, 164.3, 157.6, 104.9, 100.7, 97.0,

94.9, 89.3, 81.4, 56.9, 56.1, 34.9, 33.3, 32.5, 32.4, 23.9 (2C) 14.3; $[\alpha]_D^{20} = +188^\circ$ ($c = 0.5$ in CHCl_3); HRMS (ESI $^+$) calcd for $[\text{M}+\text{H}]^+$ $[\text{C}_{21}\text{H}_{23}\text{ClO}_6]^+$ 407.1261, found 407.1259. Anal. Calcd for $\text{C}_{21}\text{H}_{23}\text{ClO}_6$: C, 61.99; H, 5.70, found: C, 62.02; H, 5.73.

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Supporting Information Available

Full experimental data for compounds **3**, **39-41** and **44** as well as copies of NMR spectra for compounds **3**, **7**, **8**, **15**, **19**, **21**, **22**, **26**, **28-35** and **38-44**. This material is available free of charge via the internet at <http://pubs.acs.org>.

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Synthesis and Single Crystal X-ray Analysis of two Griseofulvin metabolites

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The supporting information from Chapter 5 can be seen in Appendix E



Synthesis and single crystal X-ray analysis of two griseofulvin metabolites

Mads H. Rønne^{a,b}, Pernille Harris^a, Charlotte H. Gotfredsen^a, Thomas O. Larsen^b, Mads H. Clausen^{a,*}

^a Department of Chemistry, Technical University of Denmark, Kemitorvet, Building 201 and 206, DK-2800 Kgs. Lyngby, Denmark

^b Center for Microbial Biotechnology, Department of Systems Biology, Søtofts Plads, Building 221, DK-2800 Kgs. Lyngby, Denmark

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ABSTRACT

The two phenols, 6-O-desmethyl griseofulvin and 4-O-desmethyl griseofulvin are metabolites of the antifungal drug griseofulvin. Herein, we present an improved synthesis of the 6-phenol derivative, and an unequivocal proof of both structures by single-crystal X-ray analysis.

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The natural product griseofulvin (**1**) (see Fig. 1) was first isolated by Oxford et al. in 1939¹ and later shown to possess antifungal properties.² This antifungal agent is still in clinical use today³ and is the only orally administered drug approved by the Food and Drug Administration for the treatment of tinea capitis (ringworm of the scalp).⁴ Recently, griseofulvin has received renewed attention due to reports of both antiproliferative effects in cancer cells^{5–7} as well as suppression of hepatitis C replication.⁸ As a result of its notoriously low water solubility, griseofulvin is furthermore, often used as a benchmark compound in formulation studies and in the development of drug delivery systems.⁹

The metabolism of griseofulvin has been studied both in vitro¹⁰ and in vivo and reported in several publications. In addition to studies in fungi,¹¹ the in vivo metabolism of griseofulvin has been investigated in rats,¹² mice,¹³ rabbits,¹⁴ dogs,¹⁵ and man.^{16,17} Known important metabolites of griseofulvin include 6-O-desmethylgriseofulvin (**2**) and 4-O-desmethylgriseofulvin (**3**), but their structures have never been proven unambiguously. In the literature, it is commonly merely stated that the metabolites were compared with authentic samples.^{11–13,15,17} Others^{10,14} have used spectroscopic properties and melting points to identify the structures by comparing these data with earlier work.^{18–21} We present herein, the synthesis and crystal structures of both 6-O-desmethylgriseofulvin (**2**) and 4-O-desmethylgriseofulvin (**3**), which provide final verification of the structural assignments.

6-O-Desmethylgriseofulvin (**2**) was first synthesized by Arkley et al. in six steps with an overall yield of 14% (Scheme 1).²² To confirm the outcome of these transformations, the synthetic route was

reproduced and we were actually able to isolate a small amount of **2** at step three (Scheme 1). The lengthy synthesis and poor yield of this route prompted us to search for a more convenient method to access **2**. Thus, we were pleased to obtain the desired phenol in 29% yield after the treatment of griseofulvin (**1**) with LiI in pyridine at 115 °C (Scheme 2).²² The synthesis of 4-O-desmethylgriseofulvin

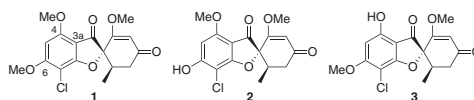
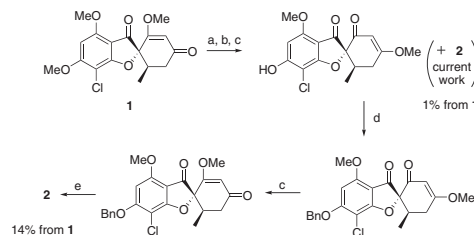


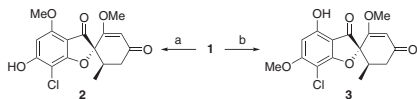
Figure 1. The structures of griseofulvin (**1**), 6-O-desmethylgriseofulvin (**2**) and 4-O-desmethylgriseofulvin (**3**).



Scheme 1. Reagents: (a) HOAc, 2 M H₂SO₄; (b) 0.5 M NaOH; (c) 2,2-dimethoxypropane, *p*-toluenesulfonic acid, MeOH; (d) K₂CO₃, BnBr, acetone; (e) 5% Pd/C, H₂, EtOAc.

* Corresponding author. Tel.: +45 45252131; fax: +45 45933968.

E-mail address: mhc@kemi.dtu.dk (M.H. Clausen).



Scheme 2. Reagents: (a) Lil, pyridine, 115 °C, (29%); (b) MgI₂, Et₂O, toluene, (98%).

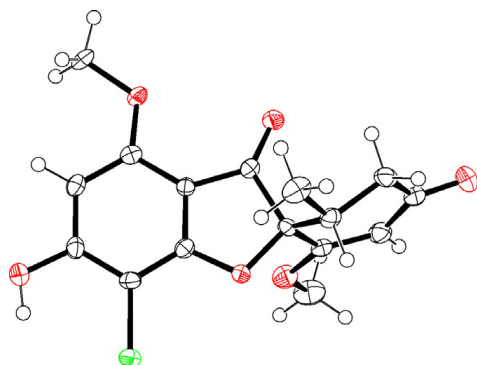


Figure 2. ORTEP view of 6-O-desmethylgriseofulvin (2).

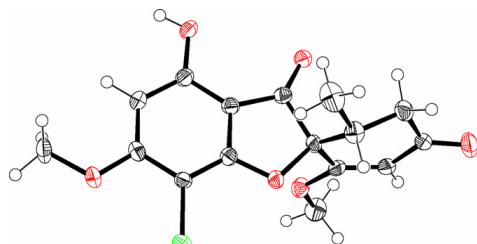


Figure 3. ORTEP view of 4-O-desmethylgriseofulvin (3).

(3) was performed by treatment of **1** with MgI₂ in a mixture of diethyl ether and toluene (Scheme 2), a slight modification of the procedure originally published by Arkley et al.²³

The structures of **2** and **3** were confirmed unequivocally by the use of single-crystal X-ray analysis (Figs. 2 and 3).²⁴ It is not possible to distinguish between the 4 and 6 methoxy groups of **1** by gHMBC as no ⁴J correlation is observed. The ¹H NMR spectrum of **2** (see Supplementary data) does not exhibit a signal for the phenolic hydroxy group, due to rapid proton exchange, and thus no heteronuclear correlations can be used to aid in the assignment of the spectrum. For **3**, the phenolic proton is observed (see Supplementary data) and the gHMBC contains a single ³J_{HC} correlation to C-3a,

confirming the position of the phenol. The UV and fluorescence spectra of **2** and **3** were all but identical, and despite small differences in the MS–MS spectra (see Supplementary data), the retention time²⁵ is still the most reliable and sensitive analytical method for distinguishing the two phenols.

Acknowledgment

The authors thank the Danish Research Council (ref. 274-07-0561) for the financial support.

Supplementary data

Supplementary data (experimental procedures, characterization, and purity data, HPLC traces, NMR, UV, and MS–MS spectra for compounds **2** and **3**, and crystallographic information in cif format) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2010.08.095

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- Synthesis of **2**. Griseofulvin (10 mg, 0.03 mmol) and Lil (4.7 mg, 0.04) were dissolved in pyridine (0.5 mL) and heated to 115 °C. After 16 h, the reaction was allowed to reach 20 °C and satd aq NH₄Cl (2 mL) was added. The mixture was extracted with EtOAc (3 × 3 mL), and the combined organic phases were dried (MgSO₄) and concentrated. Purification was performed on a Luna HPLC column (250 × 10 mm, 5 μm, C-18) using 5 mL/min H₂O/CH₃CN (isocratic run at 65:35, for 15 min) as the mobile phase to yield **2** (2.8 mg, 29%) as a yellow oil, which was crystallized from EtOAc and heptane.
- Crystallographic data (excluding structure factors) for the structures in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication nos. CCDC 775177 (**2**) and CCDC 775176 (**3**). Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 (0)1223 336033 or email: deposit@ccdc.cam.ac.uk).
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CHAPTER 6

A Technique for Selective *ipso*-Substitution of *ortho*-, *meta*- or *para*-Aryl Trimethylsilyl Groups with Radioactive Iodide in Quantitative Yields

To be communicated.

Supporting information from Chapter 6 can be seen in Appendix F

A technique for selective *ipso*-substitution of *ortho*-, *meta*- or *para*-aryl trimethylsilyl groups with radioactive iodide in quantitative yields.

Mads H. Rønneft,^{a,b} Felix Nissen,^c Thomas O. Larsen,^b Walter Mier,^c Mads H. Clausen^{a*}

^aDepartment of Chemistry, Technical University of Denmark, Kemitorvet, Building 201, DK-2800 Kgs. Lyngby, Denmark; ^bCenter for Microbial Biotechnology, Department of Systems Biology, Søltofts Plads, Building 221, DK-2800 Kgs. Lyngby, Denmark; ^cDepartment of Nuclear Medicine, University of Heidelberg, Heidelberg, Germany.

Correspondence e-mails: Mads H. Clausen: mhc@kemi.dtu.dk

Keywords: Thallium, radiolabeling, regioselectivity, griseofulvin.

A novel technique for the radiolabeling of either *ortho*-, *meta*- or *para*-aryl trimethylsilyl-substituted small molecules was developed. The method takes advantage of the *ipso*-directing and activating properties of a trimethylsilyl substituent on the arene. Treatment of a trimethylsilyl precursor with $\text{Ti}(\text{OOCF}_3)_3$ followed by sodium ^{125}I or ^{131}I consistently affords radioactive yields over 95% for all three regioisomers.

Introduction

The natural product griseofulvin (**1**, see Figure 1) was isolated by Oxford et al.¹ in 1939 and besides its long known anti-fungal properties^{2,3} it has recently been shown to possess anti-cancer^{4,6} as well as anti-viral properties.⁷ The 2'-benzyloxy-2'-demethoxy-griseofulvin analog (**2**), was previously found⁸ to be 25 times more active than **1**, in a phenotypic whole cell assay⁹ against the cancer cell line SCC114 (an oral squamous cell carcinoma cell line). The analog (**2**) was found to be the most potent amongst the 34 griseofulvin analogs tested.⁸

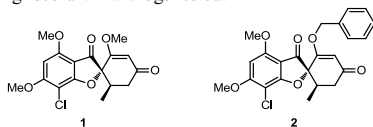


Figure 1: Griseofulvin (**1**) and 2'-benzyloxy-2'-demethoxy-griseofulvin (**2**)

The griseofulvin analog **2** warranted further investigation including $^{125}\text{I}/^{131}\text{I}$ scintigraphic *in vivo* imaging to assess bio-distribution and half-life.¹⁰ McKillop et al.¹¹ have developed a popular method for the introduction of radioactive iodide by direct electrophilic thallation of aromatic systems with $\text{Ti}(\text{OOCF}_3)_3$, followed by addition of potassium ^{125}I -iodide or ^{131}I -iodide. The substitution of the aryl thallium(III) bistrifluoroacetate with iodide affords solely the *ipso* substituted product.¹² However, the initial thallation can yield mixtures of the *ortho*-, *meta*- or *para*-thallium(III) bistrifluoroacetate products, and thus the equivalent iodo products, depending on the substrate and conditions.^{11, 13} Using a mixture of radiolabeled compounds is not satisfactory and the separation of such iodo isomers can be laborious.¹⁴ Bell et al. published a study concerning the transformation of aryl trimethylsilanes to aryl thallium(III) bistrifluoroacetates.¹⁵ It was noted that this method proceeds solely with *ipso* substitution of the TMS group. The reaction is

also efficient for deactivated aromatic substrates, while direct thallation of such compounds proceeds slowly.¹¹

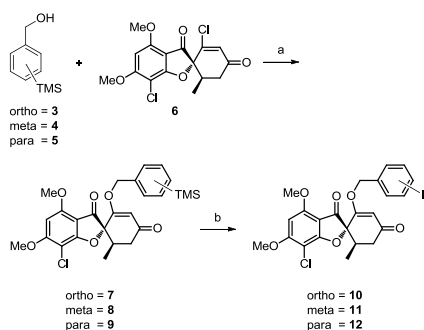
When looking for an efficient route to a radiolabeled analog of **2**, we were faced with two challenges: the instability of **2** in neat TFA, where hydrolysis to griseofulvic acid took place within minutes, and controlling the regioselectivity of the thallation. We hypothesized that combining the work of McKillop et al.¹¹ and Bell et al.¹⁵ would afford a simple route from aryl-TMS compounds to aryl iodides, via aryl thallium(III) bistrifluoroacetates, in a regioselective manner, effectively substituting the TMS group for an iodide, while taking advantage of the activating property of the silane to use milder conditions for the thallation. The activities of **10**, **11** and **12** in the phenotypic assay for multipolarity are markedly different (data not shown) and we found it prudent to develop a method that would afford a single product, while also enabling us to access either the *ortho*- (**10**), *meta*- (**11**) or *para*-iodo (**12**) isomer. Here we describe the initial results using the thallation/iodation reaction in the preparation of radiolabeled small molecules.

Chemistry

The *ortho*-, *meta*- and *para*- (trimethylsilyl)-benzyl alcohols **3**, **4** and **5** were synthesized following a known procedure¹⁶ as was compound **6**.⁸ The 1,4 conjugate addition of the alcohols to **6** was achieved with diaza(1,3)bicyclo-[5.4.0]undecane (DBU) in dioxane as previously described to yield **7**, **8** and **9**.⁸ The “cold” iodo compounds (**10**, **11** and **12**) were prepared by the same method from the corresponding *ortho*-, *meta* and *para* (iodo)-benzyl alcohols (commercially available).

The synthesis of the three “cold” iodo compounds were also completed by treating the appropriate TMS analog for 30 min. with 2 equiv. of thallium(III)trifluoroacetate in MeCN/TFA (7:3) followed by addition of excess NaI. Experiments were performed with 10, 15 and 20% TFA in MeCN, still using 2 equiv. of $\text{Ti}(\text{OOCF}_3)_3$ and excess NaI. At 10% TFA the iodo product was only observed in trace amounts but at 15 and 20% the conversion to the iodo product was 6 and 14%, respectively. It was also

investigated how 0.9 equiv of $\text{Ti}(\text{OOCF}_3)_3$ in MeCN/TFA (7:3) affected the reaction and under these conditions the conversion from **7**, **8** and **9** to the iodo products was trace amounts, 7 and 24%, respectively. The “hot” thallation/iodation was performed by treating a substrate with 50 mol% of $\text{Ti}(\text{OOCF}_3)_3$ for 2 min. followed by addition of sodium ^{125}I -iodide or ^{131}I -iodide and after 30 sec. the mixture was purified by HPLC to afford the radiolabeled compounds.



Scheme 1 (a) DBU, dioxane; (b) $\text{Ti}(\text{OOCF}_3)_3$, NaI, MeCN/TFA (7:3).

Results and Discussion

The “cold” synthesis of the iodo compounds **10**, **11** and **12** from the corresponding TMS precursors by thallation/iodation were completed on a 10 mg scale. The identity of these iodo products were confirmed by comparison (NMR, MS and retention time) with **10**, **11** and **12** prepared by the known method.

The subsequent thallation experiments were performed on a 1 mg scale and analyzed by HPLC-DAD-MS using the starting materials and verified iodo products as standards. The ratio of starting material to product is based on the absorption of each peak. All experiments were performed with a 2 min. reaction time for the thallation step and 1 min. for the introduction of iodine, which was added in excess.

Treatment of **1** with $\text{Ti}(\text{OOCF}_3)_3/\text{NaI}$ in TFA hydrolyses the 2'-enol ether completely and is thus not a viable method for introduction of radioactive iodide for this compound class. Using a mixture of MeCN/TFA (7:3), in which **1** is stable, with the same conditions resulted in no conversion. However, treating the *para*-TMS compound (**9**) with 2 equiv. of $\text{Ti}(\text{OOCF}_3)_3/\text{NaI}$ in MeCN/TFA (7:3) afforded 60% conversion to the *para* iodo compound (**12**) with starting material as the only other peak. Using the same conditions for **7** and **8** afforded the iodo products in 30% and 50% yields, respectively. The reaction does not proceed without $\text{Ti}(\text{OOCF}_3)_3$.

As “hot” labeling is performed with sub molar amounts of sodium 125-iodide/131-iodide, these levels of conversions are satisfactory as the starting material and thallium(III) bistrifluoroacetate intermediates are easily separated from the desired radiolabeled compound. The experiments with

“hot” iodide consistently afforded radioactive yields of over 95%.

Conclusion

A novel technique for the introduction of radioactive iodide has been described using thallation/iodation in 30% TFA in MeCN of aryl TMS precursors. This was achieved at both the *ortho*-, *meta*- and *para*-position of the griseofulvin analog, affording the products in over 95% radioactive yields.

Experimental

^1H NMR spectra were recorded using a Varian Mercury 300 MHz spectrometer from Agilent (Santa Clara, CA, US). ^{13}C NMR spectra were recorded using either a Varian Mercury 300 MHz or a Bruker AC 200 MHz from Bruker Optics (Ettlingen, Germany). Chemical shifts were measured in ppm and coupling constants in Hz. The residual peak of the solvent CDCl_3 was used as internal reference at δ 7.27 for ^1H NMR and δ 77.00 for ^{13}C NMR spectra. IR spectra were recorded using a Bruker Alpha ATR and measured in cm^{-1} . All melting points are uncorrected. TLC was performed on aluminium sheets precoated with silica gel 60 F254 (Merck 1.05554.0001). High-resolution LC-DAD-MS was performed on an Agilent 1100 system equipped with a photodiode array detector (DAD) and coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass, Manchester, UK) with a Z-spray electrospray ionisation (ESI) source and a LockSpray probe ($M+H$ 556.2771) and controlled by MassLynx 4.0 software. LC-MS calibration from m/z 100-900 was done with a PEG mixture. Standard separation involved a LUNA 2 column with an acetonitrile (50 ppm TFA) in water gradient starting from 15% to 100% over 25 minutes with a flow rate of 0.3 mL/min.

Compounds were visualized by charring after dipping in a solution of 1% KMnO_4 , 6.7% K_2CO_3 and 0.08% NaOH in water. UV visualization was done using a Model UVGL-25 Mineralight Lamp.

All reagents and solvents were purchased from commercial suppliers and were used without further purification. Analysis of the products was performed by reversed-phase high-performance liquid chromatography (RP-HPLC) at 60 °C on a Zorbax Stable Bond C18 1.8 μm , 4.6×150 mm column (Agilent) with a gradient of 5-60% B over 120 min (flow rate 250 $\mu\text{L}/\text{min}$; solvent A: 0.1% TFA in water, solvent B: 0.1% TFA in acetonitrile). Analysis of the radio iodinated compounds were performed by RP-HPLC (Chromolith® Performance RP-18e column, 100×4.6 mm) fitted with a Raytest GABI gamma detector (Raytest GmbH, Straubenhardt, Germany) using a gradient of 0-100% B over 5 min (200 $\mu\text{L}/\text{min}$; solvent A: 0.1% TFA in water, solvent B: 0.1% TFA in acetonitrile UV-absorption = 214 nm, γ -detection).

General procedure A – Compounds 3, 4 and 5. 2-Bromobenzyl alcohol (200 mg, 1.06 mmol) was mixed with anhydrous Et_2O (2 mL) and cooled to 0 °C. BuLi

(1.87 mL, 2.25 mmol) was added drop-wise and the mixture stirred at 0 °C for 30 min. TMSCl (0.68 mL, 5.35 mmol) was added drop-wise and the solution was allowed to reach room temperature. After 20 h the reaction mixture was quenched with sat. aq. NH₄Cl (2 mL), extracted with EtOAc (3×3 mL), and the combined organic phases were dried (MgSO₄) and concentrated. MeOH (20 mL) and a drop of HOAc was added and the mixture stirred for 1 h before re-concentration. The residue was purified by column chromatography (EtOAc:heptane 1:12.5) affording the product **3**.

2-(Trimethylsilyl)benzyl alcohol (3). Yield: 100 mg (52 %) (yellow oil); R_f-value (EtOAc/heptane, 1:3): 0.32; ¹H NMR (CDCl₃, 300 MHz): δ 7.56 (1H, dd, *J* = 7.3, 1.4 Hz), 7.49 (1H, dd, *J* = 7.6, 1.4 Hz), 7.41 (1H, ddd, *J* = 7.6, 7.3, 1.4 Hz), 7.31 (1H, dt, *J* = 1.4, 7.3 Hz), 4.77 (2H, s), 2.06 (1H, s), 0.37 (9H, s) (Litt.¹⁷); ¹³C NMR (CDCl₃, 50 MHz): δ 146.1, 138.0, 134.6, 129.4, 127.6, 126.9, 65.2, 0.3 (3C) (Litt.¹⁷).

3-(Trimethylsilyl)benzyl alcohol (4). Yield: 74 mg (39 %) (yellow oil) R_f-value (EtOAc/heptane, 1:3): 0.30; ¹H NMR (CDCl₃, 300 MHz): δ 7.50 (1H, s), 7.48-7.43 (1H, m), 7.36-7.31 (2H, m), 4.65 (2H, s), 2.11 (1H, s), 0.27 (9H, s) (Litt.¹⁷); ¹³C NMR (CDCl₃, 50 MHz): δ 140.8, 139.9, 132.6, 131.9, 127.9, 127.6, 65.4, -1.2 (3C) (Litt.¹⁷).

4-(Trimethylsilyl)benzyl alcohol (5). Yield: 97 mg (50 %) (yellow oil); R_f-value (EtOAc/heptane, 1:3): 0.33; ¹H NMR (CDCl₃, 300 MHz): δ 7.54 (2H, d, *J* = 7.9 Hz), 7.37 (2H, d, *J* = 7.9 Hz), 4.69 (2H, s), 1.66 (1H, s), 0.27 (9H, s) (Litt.¹⁷); ¹³C NMR (CDCl₃, 50 MHz): δ 141.3, 139.8, 133.5 (2C), 126.3 (2C), 65.2, -1.2 (3C) (Litt.¹⁷).

General procedure B – Compounds 7, 8, 9, 10, 11 and 12.

To a solution of **6** (0.65 mmol, 1 equiv) in 1,4-dioxane (3 mL, 0.2 M) was added the desired alcohol (1.30 mmol, 2 equiv) and DBU (1.63 mmol, 2.5 equiv). The mixture was heated to 100 °C and stirred for 18 h. The mixture was then cooled to 20 °C, and excess reagent was quenched with sat. aq. NH₄Cl (30 mL). The aqueous phase was extracted with EtOAc (3×30 mL) and the combined organic phases were dried (MgSO₄) and concentrated. The residue was purified by column chromatography (heptane:EtOAc 3:2) affording the product. When possible the product was recrystallized from EtOAc/heptane.

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-(2-trimethylsilyl)-benzyl-oxy-6'-methyl-cyclohex-2'-ene-4'-one) (7). Yield: 53 mg (20 %) (yellow oil); R_f-value (toluene:CH₂Cl₂:EtOAc, 3:3:1): 0.30; IR(neat): 1708, 1663, 1612, 1589; ¹H NMR (CDCl₃, 300 MHz): δ 7.49-7.44 (2H, m), 7.32-7.14 (2H, m), 6.09 (1H, s), 5.61 (1H, s), 4.96 (1H, d, *J* = 12.1 Hz), 4.88 (1H, d, *J* = 12.1 Hz), 4.00 (3H, s), 3.95 (3H, s), 3.08 (1H, dd, *J* = 16.5, 13.4 Hz), 2.89 (1H, ddq, *J* = 13.4, 4.5, 6.6 Hz), 2.45 (1H, dd, *J* = 16.5, 4.5 Hz), 0.99 (3H, d, *J* = 6.6 Hz), 0.26 (9H, s); ¹³C NMR (CDCl₃, 50 MHz): δ 197.0, 192.2, 169.7, 169.5, 164.5, 157.7, 139.5, 137.8, 134.5, 129.4, 127.4, 127.2, 105.8, 105.2, 97.2, 90.7, 89.5, 71.0, 56.9, 56.3, 40.0, 36.5, 14.2, 0.0 (3C); [α]_D²⁰ +162° (c = 1.0 in CHCl₃); HRMS (ESI⁺) calcd for [M+H]⁺ [C₂₆H₃₀ClO₆Si]⁺ 501.1500, found 501.1509.

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-(3-trimethylsilyl)-benzyl-oxy-6'-methyl-cyclohex-2'-ene-4'-one) (8). Yield: 58 mg (32 %) (yellow oil); R_f-value (toluene:CH₂Cl₂:EtOAc, 3:3:1): 0.31; IR(neat): 1710, 1664, 1613, 1590; ¹H NMR (CDCl₃, 300 MHz): δ 7.43-7.38 (1H, m), 7.30-7.12 (3H, m), 6.10 (1H, s), 5.62 (1H, s), 4.93 (1H, d, *J* = 12.2 Hz), 4.81 (1H, d, *J* = 12.2 Hz), 4.01 (3H, s), 3.96 (3H, s), 3.06 (1H, dd, *J* = 16.4, 13.4 Hz), 2.87 (1H, ddq, *J* = 13.4, 4.4, 6.6 Hz), 2.44 (1H, dd, *J* = 16.4, 4.4 Hz), 0.98 (3H, d, *J* = 6.6 Hz), 0.20 (9H, s); ¹³C NMR (CDCl₃, 50 MHz): δ 196.9, 192.4, 169.6 (2C), 164.5, 157.7, 140.9, 133.7, 133.0, 131.2, 127.8, 127.0, 105.9, 105.1, 97.2, 90.7, 89.5, 70.8, 56.9, 56.3, 40.0, 36.4, 14.2, -1.3 (3C); [α]_D^{187°} (c = 1.0 in CHCl₃); HRMS (ESI⁺) calcd for [M+H]⁺ [C₂₆H₃₀ClO₆Si]⁺ 501.1500, found 501.1494.

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-(4-trimethylsilyl)-benzyl-oxy-6'-methyl-cyclohex-2'-ene-4'-one) (9). Yield: 86 mg (31 %) (white crystals); R_f-value (EtOAc/heptane, 5:1): 0.59; m.p. 166-168 °C; IR: 1708, 1664, 1613, 1590, 1351, 1220; ¹H NMR (CDCl₃, 300 MHz): 7.45 (2H, d, *J* = 7.7 Hz), 7.16 (2H, d, *J* = 7.7 Hz), 6.11 (1H, s), 5.58 (1H, s), 4.92 (1H, d, *J* = 12.6 Hz), 4.82 (1H, d, *J* = 12.6 Hz), 4.03 (3H, s), 3.97 (3H, s), 3.05 (1H, dd, *J* = 16.3, 13.5 Hz), 2.87 (1H, ddq, *J* = 13.5, 4.3, 6.5 Hz), 2.43 (1H, dd, *J* = 16.4, 4.3 Hz), 0.98 (3H, d, *J* = 6.5 Hz), 0.24 (9H, s); ¹³C NMR (CDCl₃, 50 MHz): δ 196.9, 192.3, 169.5 (2C), 164.5, 157.7, 140.4, 135.1, 133.5 (2C), 125.7 (2C), 105.9, 105.1, 97.1, 90.7, 89.5, 70.6, 56.9, 56.3, 39.9, 36.4, 14.2, -1.3 (3C); [α]_D^{151°} (c = 1.0 in CHCl₃); HRMS (ESI⁺) calcd for [M+H]⁺ [C₂₆H₃₀ClO₆Si]⁺ 501.1500, found 501.1494.

General procedure C – Compounds 10, 11 and 12.

Compound **9** (9.8 mg, 0.02 mmol) was dissolved in 30% TFA in MeCN (340 μL) and Ti(OOCF₃)₃ was added (21 mg, 0.04 mmol, in 210 μL 30% TFA/MeCN). After 30 min. NaI (11.7 mg, 0.08 mmol) was added and after another 2 min. the reaction mixture was quenched with sat. aq. Na₂CO₃ (2 mL). The mixture was extracted with EtOAc (3×3 mL) and the combined organic phases were dried (MgSO₄) and concentrated. The residue was purified by column chromatography (heptane:EtOAc 2:1) to afford the product **12** (8.7 mg, 80% yield).

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-(2-iodobenzoyloxy)-6'-methyl-cyclohex-2'-ene-4'-one) (10). Yield: 5.4 mg (44 %) (white crystals); R_f-value (EtOAc/heptane, 5:1): 0.48; m.p. 167-169 °C; IR (neat, cm⁻¹): 1705, 1661, 1610, 1588; ¹H-NMR (CDCl₃): δ 7.77 (1H, d, *J* = 7.8 Hz), 7.27 (1H, m), 7.12 (1H, d, *J* = 7.4 Hz), 6.97 (1H, m), 6.12 (1H, s), 5.63 (1H, s), 4.85 (1H, d, *J* = 13.3 Hz), 4.78 (1H, d, *J* = 13.3 Hz), 4.03 (3H, s), 3.97 (3H, s), 3.09 (1H, dd, *J* = 16.5, 13.4 Hz), 2.97-2.80 (1H, ddq, *J* = 13.4, 4.4, 6.6 Hz), 2.46 (1H, dd, *J* = 16.5, 4.4 Hz), 1.00 (3H, d, *J* = 6.6 Hz); ¹³C-NMR (CDCl₃): δ 196.9, 192.2, 169.5, 169.1, 164.6, 158.8, 139.2, 136.7, 129.7, 129.3, 127.8, 106.1, 105.3, 97.2, 96.2, 90.7, 89.5, 74.4, 57.0, 56.4, 40.0, 36.4, 14.2. [α]_D^{134°} (c = 1.0 in CHCl₃); HRMS (ESI⁺) calcd for [M+H]⁺ [C₂₃H₂₁ClIO₆]⁺ 555.0071, found 555.0087.

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-(3-iodobenzoyloxy)-6'-methyl-cyclohex-

2'-ene-4'-one) (11). Yield: 1.1 mg (8 %) (white crystals); R_f -value (EtOAc/heptane, 5:1): 0.45; m.p. 171–174 °C; IR (neat, cm^{-1}): 1703, 1661, 1609, 1586; ^1H -NMR (CDCl_3): δ 7.59 (1H, d, $J = 7.8$ Hz), 7.44 (1H, s), 7.13 (1H, d, $J = 7.8$ Hz), 7.03 (1H, t, $J = 7.8$ Hz), 6.13 (1H, s), 5.57 (1H, s), 4.85 (1H, d, $J = 12.5$ Hz), 4.73 (1H, d, $J = 12.5$ Hz), 4.03 (3H, s), 3.98 (3H, s), 3.08 (1H, dd, $J = 16.5$, 13.5 Hz), 2.88 (1H, ddq, $J = 13.5$, 4.5, 6.6 Hz), 2.45 (1H, dd, $J = 16.5$, 4.5 Hz), 1.01 (1H, d, $J = 6.6$ Hz); ^{13}C -NMR (CDCl_3): δ 196.7, 192.2, 169.4, 169.1, 164.6, 157.7, 137.1, 136.8, 135.2, 130.1, 125.5, 105.7, 105.0, 97.1, 94.2, 90.6, 89.6, 69.2, 56.9, 56.3, 40.0, 36.1, 14.2; Anal. Calcd for $\text{C}_{23}\text{H}_{20}\text{ClO}_6$: C, 49.80; H, 3.63. Found: C, 49.69; H, 3.68. $[\alpha]_D^{25} +96^\circ$ ($c = 1.0$ in CHCl_3); HRMS (ESI^+) calcd for $[\text{M}+\text{H}]^+$ $[\text{C}_{23}\text{H}_{21}\text{ClO}_6]^+$ 555.0071, found 555.0063.

(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-(4-iodo)benzyloxy-6'-methyl-cyclohex-2'-ene-4'-one) (12). Yield: 8.7 mg (80 %) (white crystals); R_f -value (EtOAc/heptane, 5:1): 0.48; m.p. 166–168 °C; IR (neat, cm^{-1}): 1705, 1662, 1612, 1589; ^1H NMR (CDCl_3 , 300 MHz): δ 7.60 (2H, d, $J = 8.2$ Hz), 6.90 (2H, d, $J = 8.2$ Hz), 6.10 (1H, s), 5.54 (1H, s), 4.83 (1H, d, $J = 12.4$ Hz), 4.71 (1H, d, $J = 12.4$ Hz), 4.01 (3H, s), 3.95 (3H, s), 3.03 (1H, dd, $J = 16.5$, 13.4 Hz), 2.85 (1H, ddq, $J = 13.4$, 4.5, 6.6 Hz), 2.42 (1H, dd, $J = 16.5$, 4.5 Hz), 0.96 (3H, d, $J = 6.6$ Hz); ^{13}C NMR (CDCl_3 , 75 MHz): δ 197.3, 192.6, 169.6, 164.9, 169.7, 153.0, 137.9 (2C), 134.5, 128.7 (2C), 106.2, 105.3, 97.4, 94.1, 90.9, 89.8, 70.2, 56.6, 57.3, 40.2, 36.6, 14.1; Anal. Calcd for $\text{C}_{23}\text{H}_{20}\text{ClO}_6$: C, 49.80; H, 3.63. Found: C, 49.89; H, 3.74. $[\alpha]_D^{25} +116^\circ$ ($c = 1.0$ in CHCl_3); HRMS (ESI^+) calcd for $[\text{M}+\text{H}]^+$ $[\text{C}_{23}\text{H}_{21}\text{ClO}_6]^+$ 555.0071, found 555.0070.

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Supporting Information

^1H and ^{13}C NMR spectra for compounds **3-5** and **7-12** as well as UPLC-DAD chromatograms.

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(+)-Geodin from *Aspergillus terreus*

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A modified version of the supporting information for Chapter 7 can be seen in Appendix G

Acta Crystallographica Section C

Crystal Structure
Communications

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(+)-Geodin from *Aspergillus terreus*Mads H. Rønne^{a,b}, Morten T. Nielsen,^b Blanka Leber,^c
Uffe H. Mortensen,^b Alwin Krämer,^c Mads H. Clausen,^a
Thomas O. Larsen^b and Pernille Harris^{a*}

^aDepartment of Chemistry, Technical University of Denmark, Kemitorvet, Building 201 and 206, DK-2800 Kgs. Lyngby, Denmark, ^bCenter for Microbial Biotechnology, Department of Systems Biology, Søtofts Plads, Building 221 and 223, DK-2800 Kgs. Lyngby, Denmark, and ^cClinical Cooperation Unit for Molecular Hematology/Oncology, German Cancer Research Center (DKFZ) and Department of Internal Medicine V, University of Heidelberg, Im Neuenheimer Feld 280 (TP4), 69120 Heidelberg, Germany
Correspondence e-mail: ph@kemi.dtu.dk

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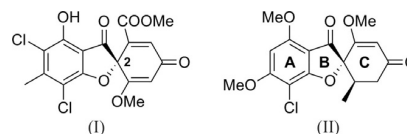
Online 23 February 2011

The fungal metabolite (+)-geodin [systematic name: (2*R*)-methyl 5,7-dichloro-4-hydroxy-6'-methoxy-6-methyl-3,4'-dioxo-spiro[benzofuran-2,1'-cyclohexa-2',5'-diene]-2'-carboxylate], C₁₇H₁₂Cl₂O₇, was isolated from *Aspergillus terreus*. The crystal structure contains two independent molecules in the asymmetric unit. Molecules denoted 1 interact through O—H...O hydrogen bonds creating chains of molecules parallel to the crystallographic 2₁ screw axis. Molecules denoted 2 interact through an O...Cl halogen bond, also creating chains of molecules parallel to the crystallographic 2₁ screw axis. Molecules 1 and 2 interact through another O...Cl halogen bond. The two molecules are similar but molecules 2 have a slightly more planar cyclohexadiene ring than molecules 1. The absolute structure of (+)-geodin has been unequivocally assigned with the spiro centre having the *R* configuration in both molecules. The structurally related (+)-griseofulvin has an *S* configuration at the spiro centre, a difference of potential biological and biosynthetic relevance.

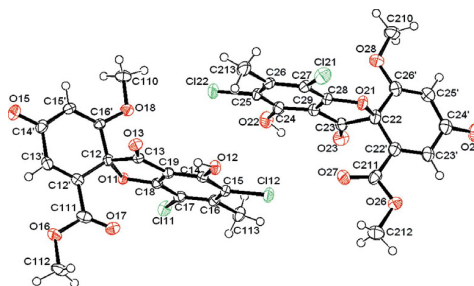
Comment

(+)-Geodin, (I), was originally isolated from *Aspergillus terreus* (Raistrick & Smith, 1936) and elucidation of its structure was initiated (Clutterbuck *et al.*, 1937; Calam *et al.*, 1939, 1947), eventually resulting in the correct relative structure (Barton & Scott, 1958). A number of biological activities have been reported for (I), including antiviral (Takatsuki, Suzuki *et al.*, 1969; Takatsuki, Yamaguchi *et al.*, 1969), antimicrobial (Rinderknecht *et al.*, 1947), enhancement of fibrolytic activity (Shinohara *et al.*, 2000) and stimulation of glucose uptake for rat adipocytes (Sato *et al.*, 2005). Furthermore, (I) is a subunit of the compound Sch 202596, an antagonist of the galanin receptor subtype GALR1 (Chu *et al.*, 1997). In an effort to synthesize Sch 202596, the total synthesis

of racemic geodin was completed (Katoh *et al.*, 2002; Katoh & Ohmori, 2000). Geodin, (I), shares the same grisan backbone as (+)-griseofulvin, (II), consisting of ring systems *A*, *B* and *C*, as shown for (II) in the Scheme below (Grove *et al.*, 1952). Additionally, both compounds (I) and (II) are dextrorotatory and this general similarity prompted our interest in (I) since (II) has anticancer properties (Ho *et al.*, 2001; Panda *et al.*, 2005). (I) was isolated from *A. terreus* and tested in our cellular anticancer assay (Rebacz *et al.*, 2007) but did not exhibit any activity (data not shown).



(I) crystallizes with two independent molecules in the asymmetric unit (Fig. 1). Although the two molecules are quite similar, there are small differences in their geometries. Cyclohexadienone ring *C* in (I) is almost planar, with an r.m.s. deviation of the least-square planes of 0.045 and 0.016 Å for molecules 1 and 2, respectively. The largest deviation from this plane is 0.070 (2)/0.024 (2) Å found for atoms C12/C22. The distances O15...O13/O25...O23 are 4.999 (3)/5.245 (4) Å, reflecting the fact that the *C* ring in (I) for molecule 2 is slightly more planar than that of molecule 1. In comparison, cyclohexenone ring *C* in griseofulvin, (II) (Puttaraja *et al.*, 1982), has a half-chair conformation, with atoms C2 and C6' on the opposite sides of the plane. This means that the distance O5...O3 is only 4.06 Å, *i.e.* much shorter than the equivalents O15...O13 and O25...O23 in (I). The ester groups in the two molecules in the asymmetric unit of (I) are rotated 21.4 (5)/16.9 (4)° with respect to the planes of the respective cyclohexadienone rings so that atoms O17/O27 are located more or less above the centre of the respective five-membered ring with short interatomic O17...O11/O27...O21 distances of 2.916 (4)/2.954 (3) Å. The five-membered *B* rings in the two molecules in (I) are rotated 89.89 (9)/88.13 (10)° with respect to the *C* rings. They are almost planar, with an

**Figure 1**

A perspective view of the two independent molecules of geodin, (I), showing the atom-numbering scheme and with displacement ellipsoids drawn at the 50% probability level.

organic compounds

r.m.s. deviation of the five atoms of 0.028/0.034 Å for both molecules. However, atoms O11(O21) and C13(C23) are below the plane and C12(C22) above the plane. In both molecules, there is an intramolecular hydrogen bond from atom O12/O22 to O13/O23 (see Table 2).

The crystal packing down the *a* axis is shown in Figs. 2 and 3, with the view showing the different packing of molecules 1 (Fig. 3*a*) and 2 (Fig. 3*b*) down the *c* axis. Molecules 1 are hydrogen bonded with a hydrogen bond from atom O12 to atom O15 in a neighbouring molecule (see Table 2, and Figs. 2 and 3*a*). This creates a chain of molecules along the crystallographic 2_1 screw axis. A similar hydrogen bond is not found in molecules 2. They are tilted slightly and the distance between atoms O22 and O25($-x + 2, y + \frac{1}{2}, -z + 2$) is 4.521 (4) Å. There are, however, halogen bonds between atoms O23 and Cl21 from neighbouring molecules (see Table 1, and Figs. 2 and 3*b*) creating chains of molecules along the crystallographic 2_1 screw axis. Furthermore, molecules of type 2 are oriented so that the *AB* ring system stacks with the *C* ring from the next molecule in the helix. Molecules 1 and 2 interact *via* halogen bonds between atoms O27 and Cl12 (see Table 1).

To increase knowledge of the structure–activity relationship of these related compounds (Rønneest *et al.*, 2009) the absolute structure of (I) presented here was determined using anomalous signal from all reflections (Flack, 1983). This showed an *R* configuration at the spiro centre of both crystallographically independent molecules. In contrast, for (II) the absolute configuration was determined based on alcoholic reactions (MacMillan, 1959) and later verified by Brown & Sim (1963) by crystal structure determination of a brominated derivative using film data to be *S* at the spiro centre and *R* at atom C6'. This structural difference between (I) and (II) could poten-

tially contribute to the observed absence of anticancer activity (Rebacz *et al.*, 2007) for (I).

Based on enzymatic studies of the biosynthesis of geodin, (I) (Fujii *et al.*, 1983), the spirocyclization reaction joining the

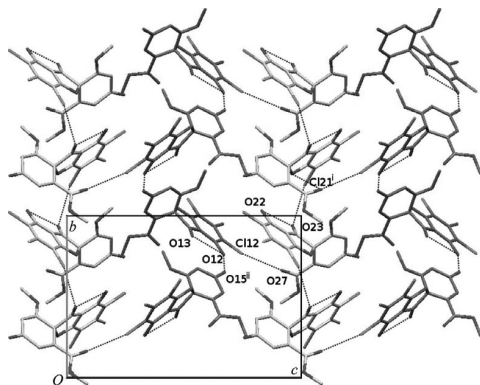


Figure 2

The molecular packing of (I), showing the hydrogen- and halogen-bond architecture; the view direction is down [100]. Hydrogen and halogen bonds are drawn as dashed lines. [Symmetry codes: (i) $-x + 1, y + \frac{1}{2}, -z + 2$; (ii) $-x, y - \frac{1}{2}, -z + 1$.]

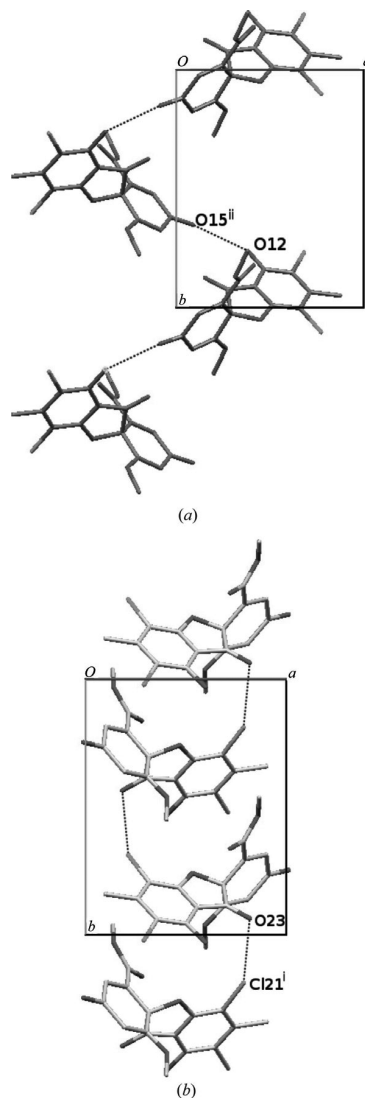


Figure 3

Helical chains in (I), viewed in the [001] direction, showing (a) molecules of type 1 and (b) molecules of type 2. [Symmetry codes: (i) $-x + 1, y + \frac{1}{2}, -z + 2$; (ii) $-x, y - \frac{1}{2}, -z + 1$.]

B and C rings is believed to be catalysed by an enzyme of the multicopper protein class. The same reaction in the griseofulvin, (II), biosynthesis, on the other hand, is presumed to be mediated by a cytochrome P450 enzyme. The latter assumption is founded on the identification of the griseofulvin, (II), biosynthesis gene cluster (Chooi *et al.*, 2010). These observations could explain the different configuration of the spiro centres of (I) and (II) since it is reasonable that enzymes of different classes lead to a disparate outcome in a similar reaction with comparable substrates.

Experimental

A. terreus [IBT 28226, culture collection at Department of Systems Biology, Technical University of Denmark (Lyngby, Denmark)] was cultured on 50 plates of yeast extract sucrose agar at 298 K for 7 d, extracted with ethyl acetate (2 l) and then concentrated to afford 1.2 g of raw extract. The raw extract was dissolved in 10% H₂O in MeOH (50 ml) and the aqueous phase was extracted with heptane (50 ml). The water content was increased to 50% by adding H₂O (40 ml) and the resulting mixture was shaken with CH₂Cl₂ (90 ml). The CH₂Cl₂ phase was concentrated (0.86 g) and further purification was performed on a Phenomenex Luna(2) HPLC column (250 × 10 mm, 5 µm, C-18) using 5 ml min⁻¹ H₂O/CH₃CN (isocratic run at 50/50 for 15 min) as the mobile phase to yield (I) (11.6 mg as a yellow oil). Geodin, (I), was crystallized using sitting-drop vapour diffusion, the drop consisting of EtOAc-heptane (4:1 v/v) and the reservoir containing heptane, to afford yellow crystals.

Crystal data

C ₁₇ H ₁₂ Cl ₂ O ₇	<i>V</i> = 1668.76 (10) Å ³
<i>M_r</i> = 399.17	<i>Z</i> = 4
Monoclinic, <i>P</i> 2 ₁	Mo <i>K</i> α radiation
<i>a</i> = 8.9276 (3) Å	<i>μ</i> = 0.43 mm ⁻¹
<i>b</i> = 11.3625 (4) Å	<i>T</i> = 120 K
<i>c</i> = 16.5006 (6) Å	0.25 × 0.15 × 0.08 mm
<i>β</i> = 94.456 (1)°	

Data collection

Bruker SMART platform CCD diffractometer	22828 measured reflections
Absorption correction: multi-scan (<i>SADABS</i> ; Bruker, 2000)	8165 independent reflections
<i>T</i> _{min} = 0.82, <i>T</i> _{max} = 0.97	7496 reflections with <i>I</i> > 2σ(<i>I</i>)
	<i>R</i> _{int} = 0.029

Refinement

<i>R</i> [<i>F</i> ² > 2σ(<i>F</i> ²)] = 0.053	H-atom parameters constrained
<i>wR</i> (<i>F</i> ²) = 0.145	Δρ _{max} = 0.81 e Å ⁻³
<i>S</i> = 1.06	Δρ _{min} = -0.36 e Å ⁻³
8165 reflections	Absolute structure: Flack (1983),
480 parameters	3819 Friedel pairs
1 restraint	Flack parameter: 0.04 (6)

H atoms were observed in a difference synthesis and subsequently placed in idealized positions. They were refined using a riding model, with aryl C—H = 0.95 Å and *U*_{iso}(H) = 1.2*U*_{eq}(C), methyl C—H = 0.98 Å and *U*_{iso}(H) = 1.5*U*_{eq}(C), and hydroxy O—H = 0.84 Å and *U*_{iso}(H) = 1.2*U*_{eq}(O).

Data collection: *SMART* and *SAINT* (Bruker, 1998); cell refinement: *SMART* and *SAINT*; data reduction: *SMART* and *SAINT*; program(s) used to solve structure: *SHELXS97* (Sheldrick, 2008); program(s) used to refine structure: *SHELXL97* (Sheldrick, 2008);

Table 1

Selected interatomic distances (Å).

O27...C112	3.070 (3)	O23...C121 ⁱ	3.006 (3)
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Symmetry code: (i) $-x + 1, y + \frac{1}{2}, -z + 2$.

Table 2

Hydrogen-bond geometry (Å, °).

<i>D</i> —H... <i>A</i>	<i>D</i> —H	H... <i>A</i>	<i>D</i> ... <i>A</i>	<i>D</i> —H... <i>A</i>
O12—H12...O15 ⁱⁱ	0.84	2.33	2.916 (3)	128
O12—H12...O13	0.84	2.50	3.149 (3)	135
O22—H22...O23	0.84	2.36	3.021 (4)	136

Symmetry code: (ii) $-x, y - \frac{1}{2}, -z + 1$.

molecular graphics: *ORTEP-3* (Farrugia, 1997) and *Mercury* (Macrae *et al.*, 2006); software used to prepare material for publication: *enCIFer* (Allen *et al.*, 2004).

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Supplementary data for this paper are available from the IUCr electronic archives (Reference: LG3050). Services for accessing these data are described at the back of the journal.

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organic compounds

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Aurantiamine from *Penicillium cavernicola*

Prior to my external stay at DKFZ I worked with the active strain *Penicillium cavernicola* (IBT 3235, CYA) and although the known diketopiperazine aurantiamine was quickly identified as a possible candidate for the activity, the isolation of aurantiamine was initiated.

This was done for a number of reasons, the inhibition of centrosomal clustering had not earlier been associated with aurantiamine, no other active compound was close to being isolated and the reason for the external stay at DKFZ was to learn about the assay and the experiments used for the initial paper by Blanka Rebacz.¹ As those experiments had already been performed with griseofulvin, repeating them again would be unproductive. On those grounds it was decided to isolate aurantiamine and bring it to DKFZ. In the following section experiments have been performed by me under the guidance of Blanka Rebacz except for the centrin staining (in Figure 8.2) and the work on the HeLa, U2OS, MDA-MB-231 and RPE cells which was performed by Simon Anderhub.

Penicillium cavernicola - aurantiamine

The alkaloid aurantiamine (**1**) was first isolated from *Penicillium aurantiogriseum*² and HPLC coupled to diode array analysis suggested that it is also produced by *P. cavernicola*.³ This has been confirmed by the isolation and identification of aurantiamine as a free base (**1**) in an extract from *P. cavernicola* (IBT 3235, CYA).

Aurantiamine (**1**) is a diketopiperazine derived from histidine and valine. Diketopiperazines have been isolated from numerous natural sources such as marine derived fungi,⁴ sponges⁵ and bacteria.⁶ They display a broad range of biological activities, including anti-fungal,⁷ anti-viral,⁸ anti-bacterial⁹ and anti-cancer activity.¹⁰

Aurantiamine is structurally similar to phenylahistin¹¹ (**2**, Figure 1), which was initially isolated from *Aspergillus ustus*. Phenylahistin has shown anti-tumor activity with IC₅₀ values in the low micromolar range against several cancer cell lines.^{11,12,13,14} Using **2** as a lead compound, an anti-cancer drug candidate (NPI-2358) has been developed and is currently in phase II clinical trials.^{15,16,17} The cytotoxic potency of **1** and **2** against P388 murine leukemia cells has been examined and phenylahistin (**2**) was reported to be forty times more active than aurantiamine (**1**).¹⁸

The IC₅₀ of **1**, for induction of multipolar mitoses, was determined to be 40 ± 4 μ M in SCC114 squamous cell carcinoma cells. Cell cycle analysis was performed

derhub. Aurantiamine was tested against HeLa (human cervical cancer), U2OS (human osteosarcoma) and MDA-MB-231 (human breast cancer), showing induction of multipolar mitosis in all three cell lines. However, when tested against another, faster proliferating, non cancerous control cell line called Retinal Pigment Endothelial (RPE) multipolar mitosis was also induced. This was further investigated by staining MDA-MB-231, SCC114 and RPE cells with centrin antibodies (to label single centrosomes/centrioles) and γ -tubulin (to label spindlepoles). It was then checked whether each pole in the multipolar cells (after incubation with aurantiamine (**1**)) also had a centrin signal, which would support the mode of action being centrosomal declustering. This was not the case, instead it was observed that the majority of cells which divide by multipolar mitosis have at least one pole without a centrin signal. So the multipolar mitoses induced by aurantiamine (**1**) is not a specific inhibition of centrosomal clustering although it is possible that it also includes inhibition of centrosomal clustering.

It was confirmed by isolation and NMR analysis that *P. cavernicola* produces aurantiamine (**1**). The IC₅₀ value for induction of spindle multipolarity in SCC114 cells for aurantiamine (**1**) has been determined to be $40 \pm 4 \mu\text{M}$. Cell cycle analyses were performed and showed that following exposure to **1**, SCC114 cells are arrested in M phase followed by apoptosis, while BJ fibroblasts were affected to a much smaller extent by aurantiamine (**1**). However RPE cells treated with **1** followed by staining against centrin and γ -tubulin showed poles with no centrosomes and thus aurantiamine (**1**) is not a specific inhibitor of centrosomal clustering.

8.1 Experimental

Solvents were purchased from Sigma-Aldrich Chemical Co. and used without further purification. 1D and 2D NMR spectra were recorded using a Varian Unity Inova 500 MHz spectrometer. Chemical shifts were measured in ppm and coupling constants in Hz. The residual peak for CDCl₃ was used as internal reference at σ 7.27 for ¹H NMR and σ 77.00 for ¹³C NMR spectra. High-resolution LC-DAD-MS was performed on an Agilent 1100 system equipped with a photodiode array detector (DAD) coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass) with a Z-spray electrospray ionisation (ESI) source, a LockSpray probe (M+H 556.2771) and controlled by MassLynx 4.0 software.

SSC114 cells were used for determining IC₅₀ value, cell cycle analysis and Western blotting. Despite the presence of supernumerary centrosomes in 64.5% of SCC114 cells, only 3.6% of the cells in mitosis form multipolar spindles. The remainder of the cell population clusters its centrosomes and undergoes bipolar mitoses, making this cell line an ideal model system.

Flow cytometry to assay cell cycle distribution: trypsinized cells were fixed in 70% aq. ethanol and stained with an antibody to phosphorylated histone H3 (Upstate Biotechnologies, 1:500) for 2 hours at room temperature, followed by a 30 min incubation with secondary antibody Alexa Fluor[®] 488 (Molecular Probes, 1:1000). Cells were then counterstained with propidium iodide and analyzed on a FACScan flow cytometer (BD Biosciences) using Cellquest software.

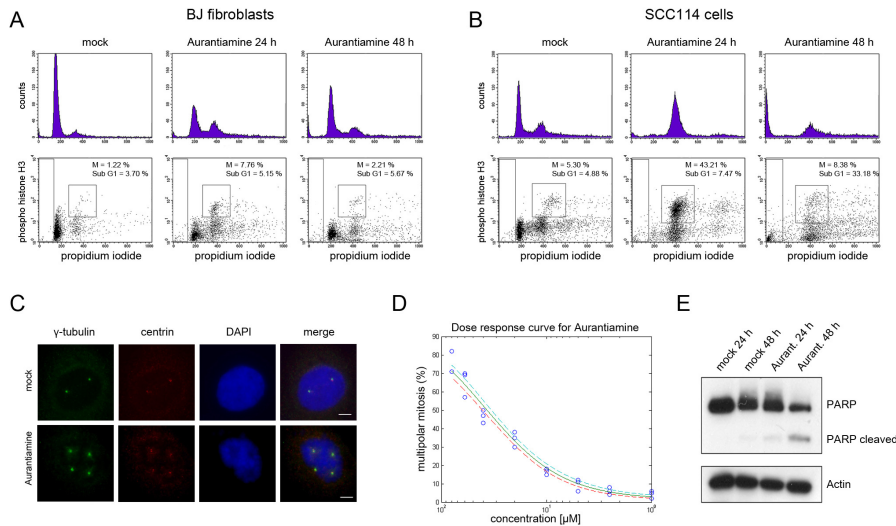


Figure 8.2: (A) FACS histograms of BJ fibroblasts treated with aurantiamine (**1**), where a slight increase of cells in mitosis can be seen. (B) SCC114 squamous cell carcinoma cells treated with **1**. After 24 hours the population in mitosis has increased significantly, while after 48 hours the sub-G1 population has increased. (C) Mock-treated SCC114 cancer cells and same cells treated with **1**. Cells were stained with an anti-centrin mouse monoclonal antibody and an anti- γ -tubulin rabbit polyclonal antibody using goat anti-mouse Cy3 and goat anti-rabbit Alexa 488 as secondary antibodies. Bipolar and multipolar mitosis can be seen, respectively. (D) Dose-response curve for SCC114 cells treated with **1**. (E) Western blot (PARP) using whole cell lysates stained with an antibody to full-length and cleaved PARP. Actin provides the loading control.

Western blot (PARP): whole cell protein extracts were prepared by lysis of cells in an appropriate volume of RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P40 (Genaxxon), 0.5% sodium deoxycholate, 0.1% SDS) supplemented with one Complete Protease Inhibitor Cocktail Tablet (Roche Diagnostics) per 50 ml of buffer, followed by mechanical homogenization and collection of the supernatant after centrifugation for 10 min at 20,000 x g. Immunoblotting was performed according to standard protocols. For detection, an antibody to full-length and cleaved PARP (Clone 9532, Cell Signaling) was used following incubation with a HRP-conjugated secondary antibody in BSA.

P. cavernicola (IBT 3235) was cultured on 200 plates of Czapek yeast autolysate agar at 25 °C for 7 days and extracted with 1% formic acid in ethyl acetate. 1.0 g of the extract (2.6 g total) was fractionated on an Isolera™ (Biotage) using a gradient from 30 to 100% acetonitrile in water over 30 minutes and a 50 gram SNAP cartridge. The fraction collected from 6.1 to 9.4 minutes was purified on a Luna HPLC column (250 × 10 mm, 5 μm, C-18) using 4 mL/min H₂O/CH₃CN (Starting at 70/30, increasing to 40/60 over 14 minutes) as the mobile phase to yield 1 (6.2 mg as a yellow oil).

Aurantiamine 1: ¹H NMR (500 MHz, CDCl₃): σ 11.99 (1H, s), 9.01 (1H, br s), 7.55 (1H, s), 6.94 (1H, s), 6.13 (1H, s), 6.04 (1H, dd, *J* = 17.3, 10.5 Hz), 5.22 (1H, d, *J* = 10.5 Hz), 5.18 (1H, d, *J* = 17.3 Hz), 4.06 (1H, t, *J* = 2.7 Hz), 2.48 (1H, d, *J* = 2.7, 7.0 Hz), 1.52 (6 H, s), 1.06 (3H, d, *J* = 7.0), 0.96 (3H, d, *J* = 7.0) (in agreement with litt.¹⁸); ¹³C NMR (from gHSQC, gHMBC, 500 MHz, CDCl₃): σ 164.7, 160.6, 144.3, 136.4, 132.1 (2C), 123.6, 113.2, 105.1, 61.0, 37.4, 32.8, 27.8 (2C), 18.5, 15.8 (in agreement with litt.¹⁸); HRMS (ESI+) calcd for [M+H] [C₁₆H₂₃N₄O₂]⁺ 303.1821, found 303.1814. [α]_D²¹ -116 (c 0.10, MeOH) (in agreement with litt.¹⁸).

Aurantiamine was tested in triplicate at 8 different concentrations (80, 60, 40, 20, 10, 5, 2.5, 1 μM). Cells were then fixed and examined by fluorescence microscopy. Two hundred mitotic cells per well were analyzed with the percentage of mitotic cells with multipolar spindles being the read-out. The relationship between readout and the dose was described by a logistic model (see equation 8.1)

$$p = \frac{1}{(1 + \exp(-\alpha - \beta x))} \quad (8.1)$$

where p is the percentage of mitotic cells with multipolar spindles and x is the dose scaled according to x=ln(dose+1). The dose axis was scaled to spread out the low concentration data. The IC₅₀ values and the corresponding 95% confidence intervals were estimated based on the fitted dose-response curves. The calculations were performed in Matlab 7.0.4.365 (The Mathworks, Natick, Massachusetts, USA) using the *glmfit* and *glmval* functions.

8.2 2'-Benzyloxy-2'-demethoxy-griseofulvin and inhibition of centrosomal clustering

The most potent compound from the initial phenotypical assay, 2'-benzyloxy-2'-demethoxy-griseofulvin (**3**, see Figure 8.3) was examined further based on the

findings with **1**. It was attempted to verify if the multipolarity was indeed inhibition of centrosomal clustering or unspecific induction of multipolarity (For the full manuscript see Appendix A). At lower concentrations **3** showed inhibition of centrosomal clustering, while at higher concentrations unspecific multipolar mitoses were observed. 2'-Benzyloxy-2'-demethoxy-griseofulvin (**3**) is therefore not 100% specific towards cancer cells, but the difference in activity between cancer cells and healthy cells provide a therapeutic window.

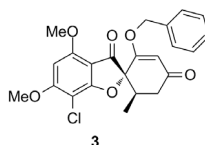


Figure 8.3: 2'-Benzyloxy-2'-demethoxy-griseofulvin **3**

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A possible way to specific anti-cancer agents is to explore the differences between healthy cells and cancer cells. One specific difference is the supernumerary centrosomes discovered in cancer cells. This affords the possibility of multipolar mitosis producing unviable progeny. To circumvent this, the cancer cells utilize a mechanism called centrosomal clustering, in order to ensure bipolar mitosis. As healthy cells contain only two centrosomes during mitosis, this mechanism is not used by healthy cells. A small molecule that inhibits centrosomal clustering and forces cancer cells to undergo multipolar mitosis, could therefore be an anti-cancer agent with specificity for cancer. Prior to this project, griseofulvin (**1**, see Figure 9.1) was identified as a small molecule that induces multipolar mitosis in cancer cells.

The chemistry of griseofulvin (**1**) was reviewed in Chapter 2, covering the areas of total synthesis, biosynthesis, microbial transformation, spectroscopic studies, and analog synthesis. More than 400 analogs have been synthesized since the discovery of griseofulvin (**1**) covering most positions of griseofulvin. The review will be of help to other researchers working in the field of griseofulvin, with its overview of more than 120 published papers concerning griseofulvin (**1**).

In Chapter 3 the structure-activity relationship study of griseofulvin analogs tested in a phenotypical assay was described. A total of 34 (nine novel) griseofulvin analogs were described with a further ten isogriseofulvin analogs tested and found inactive. The analogs covered five positions of griseofulvin (**1**), namely the 4, 5, 2', 3', and 4' positions. Elongation of the 2' position increased activity and introducing bulkier groups further improved potency with the 2'-benzyloxy (**2**) analog the most potent with an IC_{50} value of $0.9\ \mu M$. Introduction of an oxime to **1** at the 4' position increased activity slightly but did not show a cumulative effect with the 2'-benzyloxy group (**3**). Alterations at the 4 and 5 positions rendered the analogs inactive and isogriseofulvin analogs were all inactive. This work has contributed to griseofulvin's structure-activity relationship study, an area otherwise poorly examined.

In Chapter 4 55 analogs (11 novel) were tested against two dermatophytes (*Trichophyton rubrum* and *T. mentagrophytes*) as well as the cancer cell line MDA231 (human breast adenocarcinoma) in growth inhibition assays. The IC_{50} values from the phenotypical assay was found to correlate well with the values from the cytotoxicity assay ($R^2 = 0.70$). Tubulin has been proposed as the target for griseofulvin in both fungal cells and mammalian cancer cells. Tubulin is highly

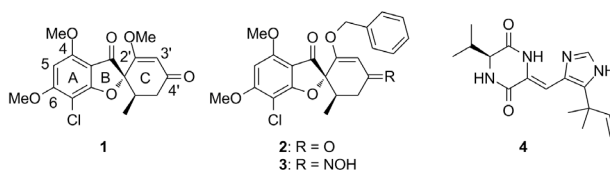


Figure 9.1: The structures of griseofulvin (**1**), 2'-benzyloxy-2'-demethoxy-griseofulvin (**2**), the 4'-oxime (**3**) of the latter and aurantiamine (**4**)

conserved in eucaryotic cells and if tubulin indeed is the target both for fungi and cancer cells, it was anticipated that the structure-activity relationship study would show the same trend, however this was not the case. The vast majority of analogs showed lower potency against the two fungi and higher potency against the cancer cell line. The 2'-benzyloxy-2'-demethoxy-griseofulvin analog (**2**) was less active against both fungi tested in this work and also less active against six dermatophytes previously tested by Crosse et al (1964). By contrast compound **2** was nine times more potent than griseofulvin (**1**) in the cytotoxicity assay, highlighting the different SAR and thus indicates different targets in fungi and mammalian cancer cells.

The accepted standards (Clinical and Laboratory Standards Institute and the European Committee on Antimicrobial Susceptibility Testing) for testing compounds against various fungi uses concentrations in mg/L, also for comparing compounds with a variety of molar masses. Visual inspection is used as a mean of evaluating minimum inhibition concentrations. The assay used for testing against fungi in this work used molar concentrations to be able to compare analogs more accurately. The minimum inhibition concentrations were based on spectroscopic measurements, which were processed to IC₅₀ values by fitting to a sigmoidal model with the software GraphPad Prism. Using a spectrophotometer as opposed to visual inspection is a more objective way of evaluating raw data. The work presented here is thus a valuable input to the science of antimicrobial susceptibility testing.

In Chapter 5 syntheses of the two phenols of griseofulvin were described. The 4-phenol was obtained in quantitative yields by treatment with MgI₂ with a yield of 29% for the 6-phenol by treatment with LiI in pyridine. The position of each phenol was determined by single crystal X-ray analysis and this proved the position of the 4-methoxy group in ¹H NMR to resonate upfield from the 6-methoxy group. This differentiation of the two methoxy groups will be of help to future synthetic work at ring A of griseofulvin.

The absolute structure of (+)-geodin, isolated from *Aspergillus terreus*, was determined in Chapter 7 by single crystal X-ray analysis. The spirocenter was found to have the *R* configuration, by using the Flack parameter. The compound shares the grisan A, B, and C rings of **1** but was tested inactive in the phenotypic assay. However, (+)-geodin was twice as potent when tested in the toxicity

assay against MDA-MB-231 cancer cells in Chapter 4. The determination of the absolute structure will be of interest to research of the biosynthesis of (+)-geodin.

The isolation of aurantiamine (**4**) from *Penicillium cavernicola* was described in Chapter 8. The compound was tested in a phenotypical assay for multipolarity inducing effects in SCC114 cancer cells (squamous cell carcinoma cells). The compound had an IC_{50} of $40 \pm 4 \mu M$ and by fluorescence-activated cell sorting (FACS) analysis it was observed that aurantiamine (**4**) induces M phase arrest in the SCC114 cells. It was also observed that the G_0 population increased upon treatment with aurantiamine, which was confirmed using Western blot analysis for the p85 fragment of poly (ADP-ribose) polymerase (PARP), a downstream target of activated caspase 3. The effect on BJ fibroblasts treated with aurantiamine (**4**) was minor, but RPE cells (Retinal Pigment Endothelial) were affected and multipolar spindles were observed. Aurantiamine (**4**) was therefore found not to be specific for cancer cells. It was investigated if **2** inhibited centrosomal clustering or like **4** induced unspecific multipolarity in both healthy cells and cancer cells. It was found that **2** at lower concentrations in fact inhibits centrosomal clustering in cancer cells, while at higher concentrations unspecific induction of multipolarity was observed. The analog affected healthy control cells to a lesser extent, providing an overall selectivity index of 10 to 30 fold. Though not truly specific **2** still provides a therapeutic window and at lower concentrations it does inhibit centrosomal clustering.

A novel method for the introduction of radioactive iodide to aromatic rings was described in Chapter 6. This method was used in scintigraphic *in vivo* imaging to assess bio-distribution and half-life of a radioactive griseofulvin analog. The treatment of an aryl trimethylsilyl with thallium(III)trifluoroacetate in 30% TFA in MeCN followed by potassium ^{125}I -iodide or ^{131}I -iodide affords the radiolabeled compound in over 95% radiochemical yield for all three regioisomers (*ortho*-, *meta*- or *para*-aryl). Since direct thallation/iodation of aryl compounds can yield all three regioisomers, this method offers a more selective approach. The *ortho*-, *meta*- or *para*-iodo-aryl of a given compound can be synthesized and tested to assess potency. The most suitable analog can then be chosen and the equivalent TMS compound synthesized. In this way the properties of the radiolabeled analog can be evaluated prior to labeling and only the desired iodo analog is produced.

Regarding future efforts to identify selective inhibitors of centrosomal clustering, the current setup with MDA-MB-231 cells as the cancer cell line screened against, and the RPE cells as controls, holds the foundation of a successful screening strategy. However, the assay is still operated fully manually and thus very time consuming. For the setup to be more successful the assay must be high throughput, making it possible to test a greater number of fungal raw extracts and e.g. synthetic libraries. The fractionation of raw extracts prior to testing should also be attempted. The use of explorative solid phase extraction could be used for initially active compounds to minimize the risk of isolating known anti-cancer compounds, although making this method and the assay compatible would take some work. Using a phenotypical assay, which can identify small molecules

that induces multipolar spindles in cancer cells, is still a rational screening approach, as finding a cancer specific compound is more likely than screening with a simple cytotoxicity assay.

APPENDIX A

GF-15, a Novel Inhibitor of Centrosomal Clustering, Suppresses Multiple Myeloma Growth *in vitro* and *in vivo*

This manuscript has been submitted to Blood.

**GF-15, a novel inhibitor of centrosomal clustering,
suppresses multiple myeloma growth *in vitro* and *in vivo***

Marc S. Raab^{1,2,3}, Iris Breitkreutz^{2,4}, Mads H. Rønne^{5,6}, Blanca Leber⁷, Thomas O. Larsen⁶, Ludmila Weiz^{3,7}, Simon Anderhub⁷, Gleb Konotop³, Patrick J. Hayden², Klaus Podar^{2,4}, Johannes Fruehauf⁸, Felix Nissen⁹, Walter Mier⁹, Uwe Haberkorn⁹, Anthony D. Ho¹, Hartmut Goldschmidt^{1,4}, Kenneth C. Anderson², Mads H. Clausen⁵,
and Alwin Krämer⁷

¹Dept. of Internal Medicine V, University of Heidelberg, Heidelberg, Germany; ²Dept. of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA; ³Max-Eder Group Experimental Therapies for Hematologic Malignancies, German Cancer Research Center, Heidelberg, Germany; ⁴National Center of Tumor Diseases, University of Heidelberg, Heidelberg, Germany; ⁵Dept. of Chemistry, Technical University of Denmark, Kgs. Lyngby, Denmark; ⁶Center for Microbial Biotechnology, Department of Systems Biology, Kgs. Lyngby, Denmark; ⁷Clinical Cooperation Unit Molecular Hematology/Oncology, German Cancer Research Center and Dept. of Internal Medicine V, University of Heidelberg, Heidelberg, Germany; ⁸Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA; ⁹Dept. of Nuclear Medicine, University of Heidelberg, Heidelberg, Germany;

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Correspondence: Alwin Krämer, Clinical Cooperation Unit Molecular Hematology/Oncology, German Cancer Research Center and Dept. of Internal Medicine V, University of Heidelberg, Im Neuenheimer Feld 581, 69120 Heidelberg, Germany, Phone: +49-6221-42-1440; Fax: +49-6221-42-1444, Email: a.kraemer@dkfz.de

Abstract

In contrast to normal cells, malignant plasma cells are highly aneuploid and frequently contain multiple centrosomes. To allow for bipolar mitotic division, supernumerary centrosomes are clustered into two functional spindle poles in many cancer cells. Recently, we have shown that griseofulvin forces tumor cells with supernumerary centrosomes to undergo multipolar mitoses resulting in apoptotic cell death. Here, we describe the characterization of the novel small molecule GF-15, a derivative of griseofulvin, as inhibitor of centrosomal clustering in myeloma cells. In a wide array of myeloma cell lines, the mean inhibitory concentrations (IC₅₀) for proliferation and survival were found to be in the range of 1-5 μ M, associated with activation of caspases 8, 9, and 3. GF-15 overcomes the growth advantage conferred by bone marrow stromal and endothelial cell-myeloma co-culture. In addition, treatment with GF-15 was associated with inhibition of VEGF- and IGF1-triggered myeloma cell migration. Importantly, treatment of mouse xenograft models of human myeloma with GF-15 resulted in tumor growth inhibition and significantly prolonged survival. These results demonstrate the *in vitro* and *in vivo* anti-tumor efficacy of a prototype small molecule inhibitor of centrosomal clustering and strongly support the further evaluation of this new class of molecules.

Introduction

Although recent advances in the therapeutic management of multiple myeloma (MM) have improved its prognosis, no curative therapy currently exists for this disorder, which is the second most commonly diagnosed hematologic malignancy in the Western world¹. MM is a plasma cell malignancy characterized almost universally by aneuploidy and chromosomal instability. In hyperdiploid MM, which accounts for approximately 50% of cases, multiple trisomies and monosomies are observed in clonal cells. Even though the mechanisms leading to chromosomal instability in MM are only beginning to be unraveled, accumulating evidence suggests that centrosome amplification might contribute to the acquisition of aneuploidy in MM²⁻⁴.

Centrosomes are small cytoplasmic organelles which consist of a pair of centrioles embedded in pericentriolar material and act as microtubule organizing centers. During mitosis, centrosomes function as spindle poles, directing the formation of bipolar spindles, a process essential for accurate chromosome segregation^{5,6}. Centrosomes duplicate precisely once per cell cycle to assure spindle bipolarity, with each daughter cell receiving one centrosome upon cytokinesis. Centrosome amplification is frequent in both solid tumors and hematologic malignancies, and is linked to tumorigenesis and aneuploidy^{2-4,7-11}. The extent of centrosomal aberrations correlates with the degree of chromosomal instability and malignant behaviour in tumor cell lines, mouse tumor models, and human tumors^{7,10,11-13}.

In mitosis, supernumerary centrosomes can lead to the formation of multipolar spindles, which is a hallmark of many tumor types⁹. Multipolar spindles, however, are antagonistic to cell viability^{14,15}. Most progeny derived from a multipolar mitosis will undergo apoptosis. To circumvent this problem, many cancer cells appear to have mechanisms that suppress spindle multipolarity, the best studied being clustering of supernumerary centrosomes into two spindle poles enabling bipolar division^{9,14-20}. Bipolar spindle formation via centrosomal clustering is associated with an increased frequency of lagging chromosomes during anaphase, thereby explaining the link between supernumerary centrosomes and chromosomal instability¹⁵.

The mechanisms of centrosomal clustering in tumor cells are incompletely understood. Recent genome-wide RNAi screens in cells containing supernumerary centrosomes suggest the involvement of the spindle assembly checkpoint, spindle

tension as controlled by cortical actin cytoskeleton and cell adhesion molecules as well as centrosome and kinetochore components in this process¹⁹⁻²¹.

Supernumerary centrosomes are almost exclusively found in a wide variety of neoplastic disorders but not in non-transformed cells. Therefore, inhibition of centrosomal clustering with consequential induction of multipolar spindles and subsequent cell death would specifically target tumor cells with no effect on normal cells with regular centrosome content^{9,17}. Using a phenotype-based screening strategy, we have recently identified griseofulvin as chemical inhibitor of centrosomal clustering in human cancer cells¹⁴. This compound induced multipolar spindles by inhibition of centrosome coalescence, mitotic arrest, and subsequent cell death in multiple tumor cell lines but not in diploid fibroblasts and keratinocytes with normal centrosome content. Chemical optimization of griseofulvin led to the development of compounds with significantly increased activity and mean inhibitory concentrations (IC₅₀) of proliferation and survival in the lower micromolar range when applied to the human squamous cell carcinoma cell line SCC114, which had been used for the initial screening²².

Here, we describe that the griseofulvin derivative GF-15 potently inhibits centrosomal clustering followed by apoptosis induction in MM both *in vitro* and *in vivo*.

Materials and methods

Materials

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(2'-benzyloxy-6'-methylcyclohex-2'-en-4'-one) (2'-benzyloxy-2'-demethoxygriseofulvin; GF-15) was synthesized following the procedures described recently²². Other reagents were obtained as follows: VEGF₁₆₅ and IGF-1 from R&D Systems (Minneapolis, MN); Caspase 8 and PARP antibodies from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture

All human MM cell lines (RPMI-8226, OPM-2, NCI-H929, OPM-1, KMS-12BM, KMS-12PE, KMS-11, KMS-18, U-266, MM1.S, LR5, Dox40, MM1.R, PAT1) and primary patient MM cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Harlan, Indianapolis, IN), 100 U/mL penicillin, 10 µg/mL streptomycin, and 2 mM L-glutamine (Cellgro, Herndon, VA). Leukemia lines used were HEL, MOLM14, and Ku812 and cultured as described above. Solid tumor cell lines comprised HeLa (cervical carcinoma), HT29, HCT116, SW480 (colorectal carcinoma), PANC1, PACA1 (pancreatic carcinoma), and LN229 (glioblastoma). HS4, KM105, KM104 (bone marrow stromal cells), THLE3 (liver cells), peripheral blood mononuclear cells (PBMC), and primary bone marrow stromal cells (BMSC) served as non-malignant controls. All solid tumor cell lines and non-malignant controls were grown in DMEM medium supplemented with 10% heat-inactivated FBS (Harlan, Indianapolis, IN), 100 U/mL penicillin, 10 µg/mL streptomycin, and 2 mM L-glutamine (Cellgro, Herndon, VA).

Isolation of patient tumor cells

After patients provided informed consent in accordance with the Declaration of Helsinki and approval by the Institutional Review Board of the University of Heidelberg, MM patient cells (96% CD38⁺ CD45RA⁻) were obtained as described²³.

Cell lysis and immunoblotting

Cell lysis and Western blot analysis were performed as described previously²³.

Evaluation of cell viability

Cell viability was examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT; Sigma Chemical, St Louis, MO) colorimetric assay, as previously described²⁴. Briefly, cells were plated in 96-well microtitre plates at a density of $2-3 \times 10^4$ cells per well, and each plate was incubated for 24 and/or 48 h, with MTT added to each well for at least 4 h. The absorbance of each well was measured at 570/630 nm using a spectrophotometer (Molecular Devices, Sunnyvale, CA). Each condition was analyzed in at least three replicates, and the results are presented as the mean \pm standard deviation of replicates of a representative experiment that was repeated at least three times.

DNA synthesis and cell proliferation assay

Cell proliferation was assessed by measuring [³H]-thymidine uptake, as described in prior studies²³.

Measurement of caspase 3/7 activation

Caspase 3/7 activation was analyzed using the Apoptosis Detection Kit from Promega according to the recommendations of the manufacturer.

Flow cytometry

Cell cycle analysis by flow cytometry including the quantification of cells in mitosis by phospho-S10-histone H3 staining was done as previously described¹⁴.

Transwell migration assay

Cell migration was assayed using a modified Boyden chamber assay, as described previously²³.

Immunofluorescence

Immunofluorescence staining was done as described²⁵. The following fluorochrome-conjugated secondary antibodies were used: anti-rabbit Alexa 488 (Molecular Probes) and anti-mouse Cy3 (Jackson ImmunoResearch Laboratories). Immunostained cells were examined using a Zeiss Axiovert 200 M fluorescence microscope. Images were processed with Photoshop software (Adobe).

MM xenograft mouse model

To determine the *in vivo* anti-MM activity of GF-15, beige-nude Xid mice were inoculated s.c. in the right flank with 3×10^6 OPM2 cells in 100 mL RPMI 1640 medium, together with 100 mL matrigel (Becton Dickinson Biosciences, Bedford, MA). When a tumor was measurable, mice were assigned to a GF-15 treatment group or the control group. GF-15 was dissolved in 100% DMSO and given daily five times a week by i.p. injection for indicated periods. The control group received the carrier alone at the same schedule and route of administration. Tumor burden was measured every alternate day using a calliper (calculated volume = $4\pi/3 \times (\text{width}/2)^2 \times (\text{length}/2)$). Animals were sacrificed when their tumor reached 2 cm or when the mice became moribund. Survival was evaluated from the first day of treatment until death. All animal studies were approved by the Dana-Farber Animal Care and Use Committee.

Preparation of the radiolabeled GF-15 analogue

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-(4-(125/131-iodo)benzyloxy)-6'-methyl-cyclohex-2'-ene-4'-one) was prepared by thallation-iodination with ^{125}I -iodide or ^{131}I -iodide (Perkin Elmer, Dreieich, Germany) of (2*S*,6'*R*)-(7-chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(6'-methyl-2'-(4-trimethylsilylbenzyloxy)-cyclohex-2'-ene-4'-one), which was in turn synthesized from *p*-trimethylsilylbenzyl alcohol²⁶ using a known method²². The radiolabeled analogue was compared to a sample of (2*S*,6'*R*)-(7-chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-(4-iodobenzyloxy)-6'-methyl-cyclohex-2'-ene-4'-one) prepared from *p*-iodobenzyloxy alcohol²² and found to be identical by HPLC-DAD-MS.

^{125}I scintigraphic *in vivo* imaging

For imaging studies, 200 μL of a solution of the ^{125}I -labeled GF-15 analogue (5 MBq/mice) was injected into the tail vein of six week old female NMRI mice. Scintigraphic images were taken using a gamma camera (Biospace, France). The accumulation of the radioactive tracer was monitored by static planar images at 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h and 24 h after injection.

Biodistribution studies

¹³¹I-labeled GF-15 analogue (1 MBq/mice) was injected via the tail vein of six week old female NMRI mice. At the time points specified, the animals were sacrificed, weighed and dissected. Organs or tissues were blotted dry and weighed. The radioactivity was measured in a γ -counter along with a sample of the injection solution to calculate the percentage of injected dose per gram of tissue (%ID/g).

Stability experiments

The serum stability was determined by incubation of the ¹²⁵I-labeled GF-15 analogue in human serum at 37 °C. Aliquots were taken at several points in time, and the degradation was stopped by precipitation of the serum proteins with acetonitrile. After 30 min at 0 °C and a further centrifugation step the clear supernatant was analyzed by reverse-phase HPLC on a Chromolith Performance RP-18e 100 × 4.6 mm column using water and acetonitrile containing 0.1% trifluoroacetic acid as the eluent.

Statistical analysis

Statistical significance of differences observed in GF-15-treated versus control cell cultures and mice was determined using an unpaired Student's *t* test. Overall survival in animal studies was measured using the Kaplan-Meier method. (* *P*>0.01; ** *P*>0.001)

Results

GF-15 leads to multipolar mitosis induction in the upper nanomolar range

Recently, we have shown that especially 2'-modified derivatives of griseofulvin have an enhanced capacity for multipolar mitosis induction as compared to griseofulvin itself²². Initial testing for the ability to inhibit centrosomal clustering was performed in SCC114 cells, an oral squamous cell carcinoma line showing pronounced centrosome amplification^{14,18}. GF-15 (2'-benzyloxy-2'-demethoxygriseofulvin,) is significantly more potent with regard to the induction of spindle multipolarity than griseofulvin (Figure 1A). The EC₅₀ value of multipolar spindle induction for GF-15 was 900 nM, corresponding to a 27-fold increased activity compared to griseofulvin. Importantly, SCC114 cells that became resistant to GF-15 after long-term culture with increasing doses (0.2 – 1 μ M) of the compound over a period of ten weeks, showed significantly less centrosome amplification and formed fewer multipolar spindles upon treatment with therapeutic doses (5 μ M) of the drug (Figure 1B, Figure S1).

To test for the contribution of centrosome declustering to total multipolar mitosis induction after treatment with GF-15, the PC-3 prostate carcinoma cell line, which harbors supernumerary centrosomes in $28 \pm 4\%$ of the cells, was treated with increasing concentrations of the drug for 24 h (Figure 1C). Intriguingly, at the highest analyzable concentration (1.5 μ M), GF-15 induced centrosome declustering – as determined by the detection of two centrioles at each spindle pole (Figure 1D) – in $27 \pm 4\%$ of the cells, thereby closely matching the total percentage of cells with supernumerary centrosomes. At the lowest concentration tested (0.375 μ M), multipolarity induction was mostly due to inhibition of centrosomal clustering. With increasing doses of GF-15, the contribution of multipolarity induction by other means in cells without supernumerary centrosomes gradually increased. From these results, it may be concluded that mechanisms responsible for holding supernumerary centrosomes together might be similar to the forces that bundle microtubules into a bipolar spindle array in cells with a regular centrosome content. However, different sensitivities of both mechanisms provide a window of opportunity to preferentially target centrosome clustering at certain dose levels.

GF-15 is particularly active against MM cells

Next, we examined the effect of GF-15 on the growth of several different cancer cell lines. GF-15 exhibits potent cytotoxicity in a concentration-dependent manner against a broad spectrum of tumor cell types including colon, cervix, glioblastoma, pancreas, leukemia, and myeloma-derived cell lines (Figure 2A). As compared to solid tumor cell lines, MM cell lines were particularly susceptible to the cytotoxic and anti-proliferative effect of GF-15 with IC_{50} values ranging from 1 - 2.5 μ M, including MM cell lines resistant to doxorubicin (Dox40), melphalan (LR5), and dexamethasone (MM1.R) (Figure 2 A, B). Moreover, primary MM cells freshly isolated from the bone marrow of three patients with relapsed myeloma showed marked cytotoxic effects upon treatment with GF-15. This was in stark contrast to their corresponding BMSCs, which displayed virtually no cytotoxicity when exposed to GF-15 (Figure 2C). Similarly, GF-15 did not induce significant cytotoxicity in PBMCs from healthy volunteers even after stimulation with phytohemagglutinin (PHA), providing an overall selectivity index of 10- to 30-fold when compared to IC_{50} values of cancer cell lines (Figure 2D). These data suggest that GF-15 exhibits both potent and selective cytotoxicity against malignant cells.

Induction of spindle multipolarity, mitotic arrest and apoptosis by GF-15

GF-15 induced multipolar mitoses in MM cell lines NCI H929, OPM2, and RPMI 8226 (Figure 3A). After 24 h of treatment with 3 μ M GF-15, no significant induction of spindle multipolarity could be detected in primary BMSCs, whereas >80% of mitoses were multipolar in NCI H929, OPM2, and RPMI 8226 cells (Figure 3B). To examine the effect of multipolar mitosis induction on cell cycle progression, starvation-synchronized OPM2 cells were exposed to GF-15, stained with propidium iodide, and subsequently analyzed by flow cytometry. GF-15 induced a pronounced G₂/M cell cycle arrest within 12 h of treatment followed by an increase of the sub-G₁ population compared to mock-treated cells (Figure 3C, upper panel). Indicating induction of apoptosis, the increase of the sub-G₁ population was concentration-dependent (Figure 3C, lower panel), analogous to the effect of griseofulvin in SCC114 cells¹⁴. To further verify apoptotic cell death triggered by GF-15, protein profiling in GF-15 treated MM cells showed dose-dependent cleavage of caspase 8, caspase 9, caspase 3, and PARP (Figure 3D). Cleavage fragments of these proteins became detectable at 0.5 μ M and strongly increased at 3 μ M of GF-15. Importantly, exposure

of primary BMSCs to 3 μ M of GF-15 for 24 h did not induce activation of effector caspases 3 and 7 compared to OPM2 cells (Figure 3E).

Evaluation of combinations of GF-15 with other anti-myeloma agents

Clinical experience in the therapeutic management of MM patients supports the notion that drug combinations can induce higher response rates when compared with single-agent treatment^{1,24}. We therefore evaluated the effects of combinations of GF-15 with other established anti-MM drugs on the viability of MM cells. Specifically, GF-15 was combined with conventional agents (melphalan, dexamethasone) as well as with more recently developed compounds such as bortezomib. While GF-15 together with dexamethasone or bortezomib, respectively, resulted in at least additive effects (data not shown), the combination with melphalan led to a marked abrogation of GF-15 induced cytotoxicity (Figure 3F). This is consistent with an S-phase arrest induced by the DNA-damaging drug melphalan, thereby preventing entry into mitosis of melphalan-exposed cells. This underlines the specificity of GF-15 for cells in G₂/M-phase and provides important information for its possible use in clinical application.

GF-15 inhibits myeloma cell growth triggered by bone marrow stromal cells

In addition to the autocrine/paracrine effects mediated by growth factors and cytokines within the MM bone marrow microenvironment, direct MM-BMSC contact also triggers tumor cell growth. We therefore evaluated the effect of GF-15 on MM cell proliferation induced by the stimulatory effect of BMSCs (Figure 4A, B). Binding of OPM2 or RPMI-8226 cells to primary BMSCs triggered increased MM cell proliferation, which was completely abrogated by GF-15. Importantly, as also shown in Figure 2C, GF-15 did not affect the viability of BMSC lines, as determined by MTT assays.

GF-15 inhibits cytokine-induced myeloma cell migration

Migration activity is required for homing of tumor cells to the bone marrow, expansion within the bone marrow microenvironment, and tumor cell egress into the peripheral blood^{23,27}. Since centrosomes and microtubules as targets of GF-15 are not only important for mitotic spindle formation but also for interphase cell shape and migration²⁸, we next investigated whether vascular endothelial growth factor (VEGF)- and insulin-like growth factor-I (IGF-I)-induced migration of MM cells can be

abrogated by pre-treatment with GF-15. Our data show that GF-15 completely abrogates both VEGF/FN- (fibronectin) and IGF-I-triggered MM-cell migration (Figure 4C).

GF-15 is rapidly eliminated *in vivo*

Derived from its parental molecule griseofulvin, GF-15 has been modified at the 2'-position (Figure 1A). We therefore sought to analyze *in vivo* stability and pharmacokinetics of this new compound. By introducing a *p*-iodobenzyl group in the 2'-position of the griseofulvin molecule we generated a ¹²⁵I-labeled GF-15-analogue. HPLC analysis showed only minimal degradation of this molecule in human serum with a half-life of 48 h at 37 °C. The cleavage products resulting from degradation are presumably 4-iodobenzyl alcohol and griseofulvic acid, consistent with the analogue undergoing hydrolysis (data not shown). After i.v.-application of trace amounts of this analogue, rapid renal clearance was observed within the first 6 h after injection (Figure 5). In light of the clearance data and the poor solubility of GF-15 at higher concentrations, we went on to investigate its *in vivo* efficacy after intraperitoneal (i.p.) application.

GF-15 exhibits *in vivo* anti-MM activity in a myeloma xenograft mouse model

In view of the potent and selective *in vitro* activity of GF-15 against MM cell lines and freshly isolated primary MM patient cells, we next examined the *in vivo* effect of GF-15 on human MM growth in immunodeficient mice. A cohort of 30 immunodeficient beige-nude-Xid (BNX) mice was inoculated with 3×10^6 OPM2 cells s.c. in the right flank. Treatment with a daily dose of 20 mg/kg (ten mice) or 100 mg/kg (ten mice) i.p. five days per week for two weeks was started when tumors became palpable. Ten mice served as a control cohort and received i.p. injections of the vehicle alone. GF-15 treatment decreased tumor growth in both cohorts of treated mice with a greater effect in the group that received 100 mg/kg i.p. (Figure 6A). Kaplan-Meier and log-rank analysis revealed a significant prolongation of survival for the GF-15 treated groups compared with the vehicle-treated controls (log-rank $P < 0.001$; Figure 6B). The toxicity profile of GF-15 seems to be quite favourable as body weight was not affected by treatment with GF-15 compared with untreated controls (Figure 6C). Importantly, examination of histological tumor sections revealed a dose-dependent,

significant increase of aberrant and multipolar mitoses in the GF-15-treated mice compared with controls ($P < 0.01$ for 20mg/kg, $P < 0.001$ for 100mg/kg; Figure 6D, E).

Discussion

Here, we have shown that treatment of MM cells with GF-15, a derivative of griseofulvin, leads to multipolar spindle formation, centrosomal declustering, mitotic cell cycle arrest, and apoptosis both in tissue culture and in a myeloma xenograft mouse model.

MM, presently an incurable plasma cell malignancy, accounts for approximately 20% of deaths from hematologic malignancies and nearly 2% of deaths from cancer^{1,29}. In virtually all cases, it is preceded by a premalignant tumor called monoclonal gammopathy of undetermined significance (MGUS), which occurs in about 3% of individuals over the age of 50³⁰. Virtually all MGUS and MM tumor cells have numeric and/or structural chromosome abnormalities and are characterized by gross chromosomal instability^{1,31}. Using gene expression profiling, we and others have shown that genes relevant for cell cycle progression and chromosomal instability are overexpressed in high-risk MM patients^{32,33}.

Centrosome amplification is common in MM and already present in MGUS²⁻⁴. Moreover, its magnitude constitutes an independent prognostic factor in both newly diagnosed and relapsed MM^{2,3}. Mutation or misregulation of a variety of tumor suppressors or oncogenes has been shown to disrupt the normal function and numeral integrity of centrosomes, among them aurora-A, a protein often overexpressed in high-risk MM³³⁻³⁵.

Recent genome-wide RNAi screens in cells containing supernumerary centrosomes suggest that only an intact spindle assembly checkpoint allows for sufficient time in order for centrosomal clustering to occur and that spindle tension is necessary for clustering of supernumerary centrosomes into a bipolar mitotic spindle array^{19,20}. In this regard, it is interesting to note that gene expression profiling identified overexpression of key regulators that normally maintain faithful segregation of chromosomes, including MAD2, BUBR1, ZWINT, PRC1, survivin, and aurora-B, both as part of a high-risk 15-gene signature in MM and a 70-gene signature of

chromosomal instability associated with poor clinical outcome in multiple cancer types^{32,36}.

Microtubule-interacting drugs are effective in the treatment of both newly diagnosed and relapsed/refractory MM, with clinical activity having been demonstrated for both microtubule-depolymerizing (vincristine) and microtubule-stabilizing agents (paclitaxel, epothilone B)³⁷⁻³⁹. Also, inhibition of the mitotic kinesin Eg5 effectively kills myeloma cells *in vitro*⁴⁰. The limited success of paclitaxel in the treatment of MM seems to primarily reflect the fact that it serves as a substrate for the MDR1/Pgp drug efflux pump. The mechanisms by which microtubule-interacting drugs induce cell death are still not completely clear⁴¹. Importantly, aside from the formation of stable microtubule bundles associated with G₂/M cell cycle arrest and increased microtubule polymer mass at high concentrations, taxanes have been described to affect centrosome organization with subsequent mitotic catastrophe at low nanomolar concentrations⁴².

Griseofulvin has been used for many years for the treatment of dermatophyte infections⁴³. Mechanistically, it inhibits mitosis in sensitive fungi⁴⁴ and mammalian cells^{45,46} but whether mitotic arrest is a consequence of microtubule depolymerization or some other action on microtubules in both fungi and human cells is still unclear^{47,48}. Although griseofulvin has been reported to bind to mammalian brain tubulin and to inhibit microtubule polymerization *in vitro*, it does so only at concentrations significantly higher than those needed for spindle multipolarity induction in cancer cells with extra centrosomes⁴⁸. Also, whether griseofulvin binds to tubulin directly or to microtubule associated proteins remains conflicting⁴⁸⁻⁵¹. Already more than 30 years ago it was reported that griseofulvin treatment induces spindle multipolarity with each mitotic center containing two centrioles in HeLa cells⁴⁵. While at lower concentrations the drug leads to multipolar spindles with centrosomes at each pole in cells with extra centrosomes, at higher concentrations spindle multipolarity with acentrosomal spindle pole formation is additionally induced. This is consistent with the concept that clustering extra centrosomes in cancer cells might be similar to focusing microtubules into a bipolar spindle array in normal cells. For detailed mechanistic understanding, it will be important to clearly determine the sequence of events: does the drug at low concentrations indeed cause declustering of supernumerary centrosomes with subsequent multipolar spindle formation or does

spindle multipolarity occur first with successive distribution of centrosomes to each pole?

For GF-15, the EC₅₀ value for multipolar spindle induction was 900 nM in SCC114 cells, corresponding to a 27-fold increased activity compared to griseofulvin itself. Also, whereas griseofulvin inhibits cell proliferation only weakly with half-maximal inhibition occurring at 25 μ M⁴⁸, GF-15 led to inhibition of MM cell growth *in vitro* at IC₅₀ values of 1 to 3 μ M.

GF-15 was well tolerated and effective in a murine MM model, as evidenced by significant inhibition of MM tumor growth in mice treated with either 20 or 100 mg/kg GF-15 i.p. daily for seven days a week. Since GF-15 is only poorly soluble in water and its biological half-life is short with little drug left 1 h after administration, it is expected that medicinal chemical optimization will lead to a further improvement in the drugs potency and in this novel therapeutic strategy in general. Indeed, preliminary data suggest that another, slightly modified analogue (GF-61) with improved water solubility exerts anti-myeloma activity in our xenograft mouse model even when administered orally (our unpublished data).

In summary, we have shown that single-agent GF-15 potently inhibits MM cell growth *in vitro* and *in vivo*, overcoming both cell-intrinsic as well as cell-interaction mediated mechanisms of drug resistance. These observations, coupled with GF-15's lack of major toxicity in a preclinical mouse model and the lack of significant toxicity of griseofulvin in humans, provide the framework for further clinical development of GF-15 in particular and centrosomal cluster inhibitors in general, directed at improving patient outcome in MM.

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Conflict-of-interest disclosure

The authors declare no competing financial interests.

Authorship

M.S.R. and I.B. contributed equally to this work. Contribution: M.S.R. designed, performed, and analyzed research and wrote the manuscript; I.B. and M.H.C. designed, performed, and analyzed research; M.H.R., B.L., T.O.L., L.W., S.A., G.K., P.J.H., K.P., J.F., F.N. performed and analyzed research; W.M., U.H., A.D.H., H.G., K.C.A. analyzed data and provided expert advice; A.K. designed the project, analyzed research and wrote the manuscript.

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Figures

Figure 1

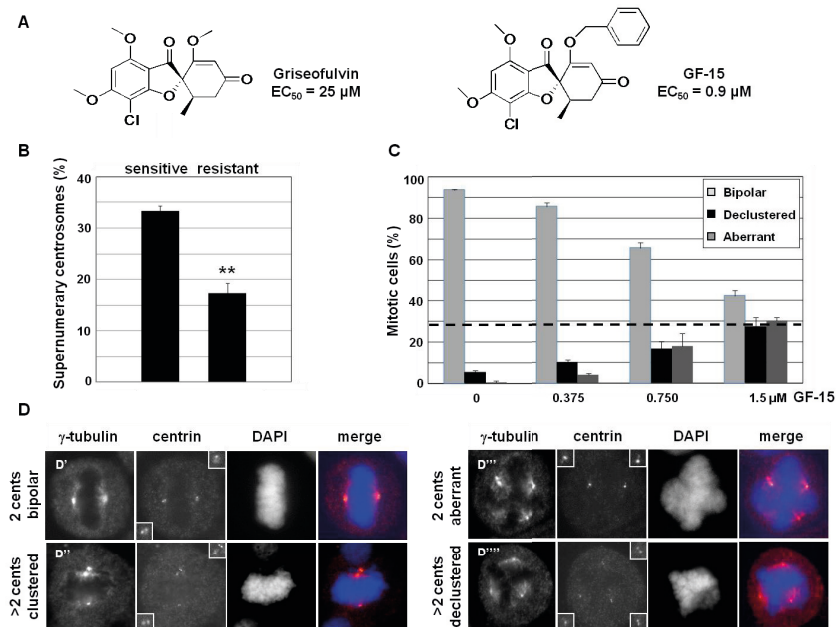


Figure 1. GF-15 is a potent and specific inducer of spindle multipolarity. (A)

Chemical structures of griseofulvin and GF-15. SCC114 cells stably expressing α -tubulin were treated with increasing concentrations of griseofulvin or GF-15 for 24 h, EC₅₀ of spindle multipolarity was assessed by immunofluorescence microscopy. (B) SCC114 cells resistant to GF-15 after long-term culture under increasing concentrations of GF-15 display significantly fewer cells with supernumerary centrosomes than wild type SCC114. Centrosomes were counted in interphase by γ -tubulin staining. (C) In PC-3 prostate cancer cells, GF-15 induces centrosomal declustering (*declustered*) in cells with amplified centrosomes, and spindle multipolarity by other means (*aberrant*) in cells with regular centrosome content in a concentration-dependent manner. The dashed line depicts the overall percentage of PC-3 cells with centrosome amplification. (D) Spindle phenotypes of PC-3 cells upon

treatment with vehicle only (D', D'') or GF-15 (D''', D''') according to their centrosome content. Cells were treated with GF-15 or vehicle only for 24 h, spindle poles were counted by γ -tubulin staining, centrioles by centrin staining.

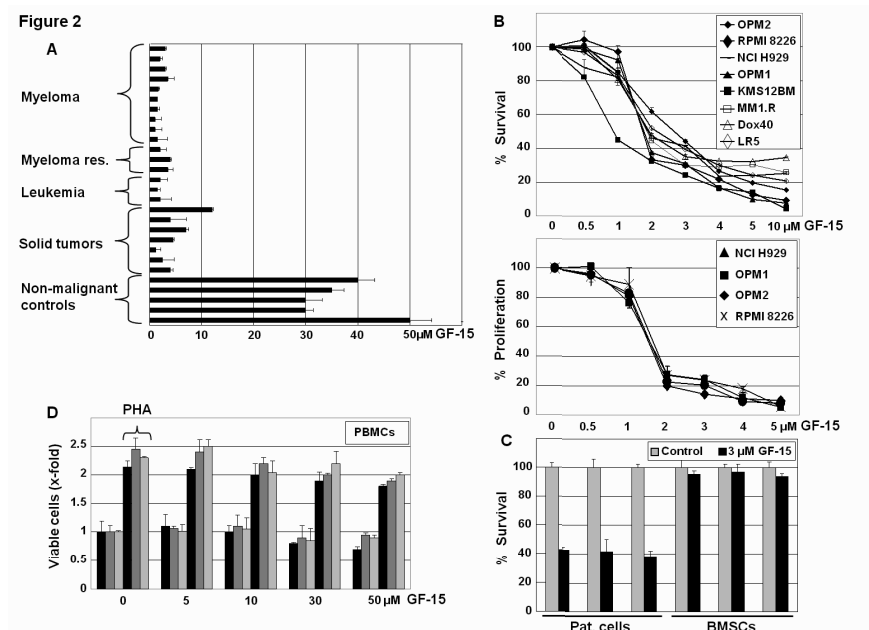
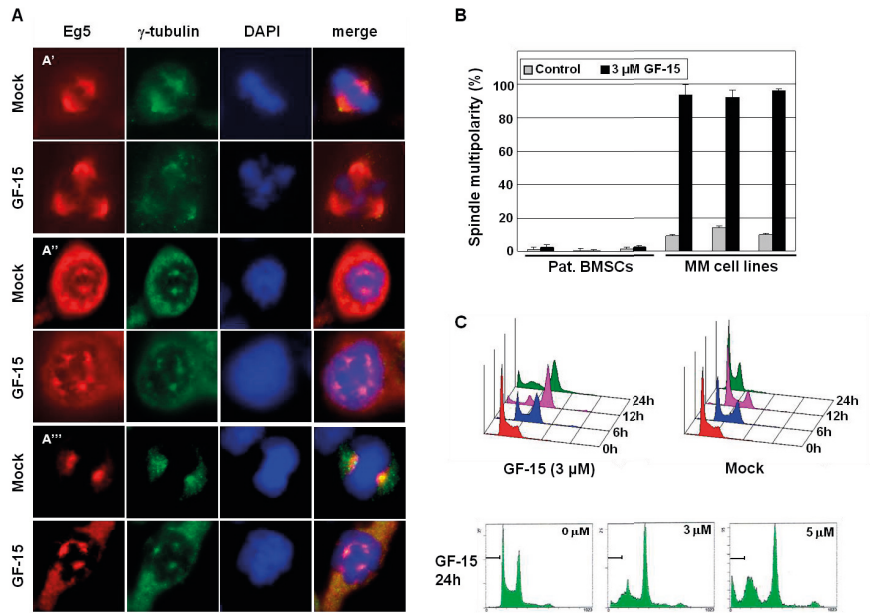


Figure 2. GF-15 selectively inhibits proliferation and survival of tumor cells. (A) IC₅₀ values of cell lines of indicated origins. (B) Dose-related effects of GF-15 on cell survival (upper panel, 48 h) and proliferation (lower panel, 24 h) on indicated MM cell lines. Cell proliferation was assessed by uptake of [³H]-thymidine during the last 8 hrs of 24 h cultures. (C) GF-15 differentially inhibits cell survival of primary cells from MM patients compared to bone marrow stroma cells (BMSCs). (D) Peripheral mononuclear cells (PBMCs) of three healthy donors activated by PHA do not show significant toxicity upon treatment with indicated concentrations of GF-15. Viable cells are expressed as x-fold of respective control. Cell survival: Unless otherwise

indicated, viable cells were measured by MTT cleavage during the last 4 hrs of 48 h cultures. Data shown are the mean +/- SD of experiments performed in triplicates.

Figure 3



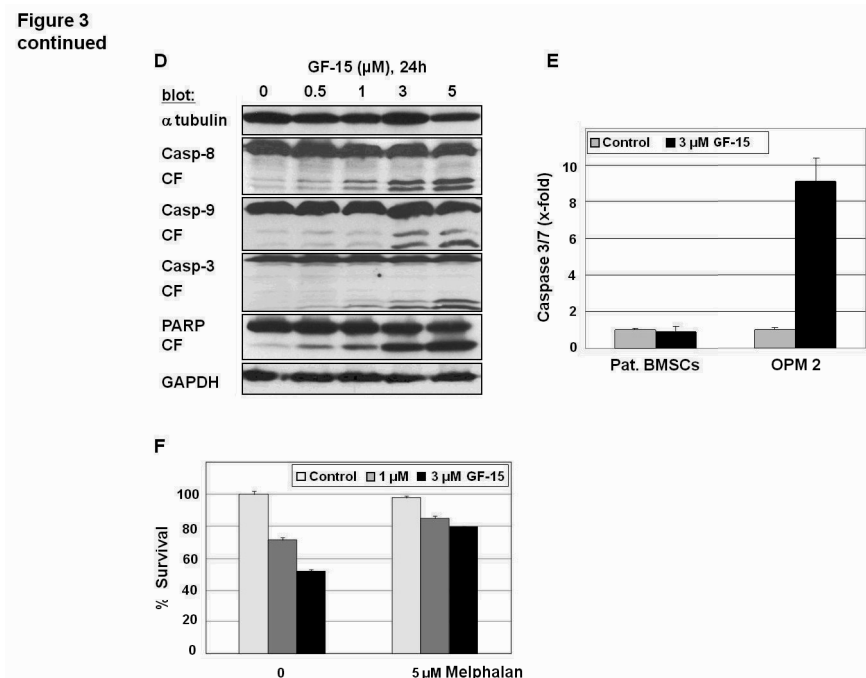


Figure 3. GF-15 specifically induces spindle multipolarity, cell cycle arrest, and apoptosis in MM cells. (A) MM cell lines display multipolar spindles upon treatment with GF-15. NCI H929 (A'), OPM2 (A''), and RPMI 8226 (A''') cells were exposed to GF-15 (3 μM ; 24 h) and stained for Eg5 (red), γ -tubulin (green), and DAPI (blue). (B) GF-15 selectively induces multipolar mitotic spindles in MM cells (NCI H929, OPM2, RPMI 8226; left to right) compared to three primary BMSCs. At least 200 mitotic cells were counted for spindle polarity after staining for γ -tubulin, Eg5, and DAPI. (C) Synchronized OPM2 cells arrest in G₂/M phase upon treatment with GF-15 followed by increase of sub-G₁ population (upper panel). Increase of sub-G₁ cells is dose-dependent (lower panel). OPM2 cells were cultured for indicated times to indicated concentrations of GF-15 and stained with propidium iodine after ethanol fixation. (D) GF-15 triggers apoptotic cell death in MM cells. OPM2 cells were exposed to

indicated concentrations of GF-15 for 24 h, followed either by immunoblot analysis of lysates with indicated antibodies or (E) ELISA-based assessment of activation of effector caspases 3 and 7. Identical treatment of BMSCs showed no significant induction of caspase 3/7 activation compared to OPM2 cells. Results are expressed as x-fold of control. (F) Co-treatment of MM cells with melphalan partially abrogates the growth inhibitory effects of GF-15. OPM2 cells were treated with indicated concentrations of GF-15 with or without 5 μ M melphalan for 48 h. MTT cleavage was measured during the last 4 hrs of 48 h cultures.

Data shown are the mean +/- SD of experiments performed in triplicates.

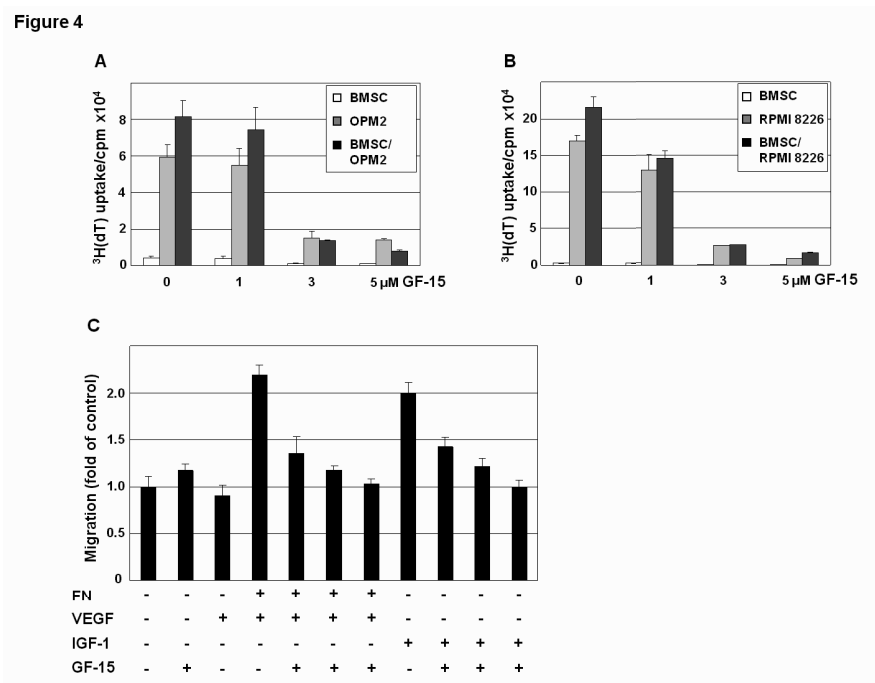


Figure 4. GF-15 abrogates the growth advantage conferred by adhesion to BMSCs, and inhibits cytokine-triggered cell migration. (A-B) Indicated MM cell lines were cultured with or without BMSCs. GF-15 was added at indicated

concentrations and proliferation was measured by [^3H]-thymidine uptake during the last 8 hrs of 24 h cultures. (C) GF-15 abrogates VEGF/FN- and IGF-1 – triggered MM-cell migration. Growth factor-deprived GF-15 treated (1, 3, 5 μM) or untreated OPM2 cells were plated on either a fibronectin-coated or non-coated membrane (8 μm pore size) in a Boyden modified chamber and stimulated with the indicated cytokines. Cells in lower chamber were counted with Coulter counter ZBII after 6 hrs of treatment. Data shown are the mean \pm SD of experiments performed in triplicates, representative of three independent experiments.

Figure 5

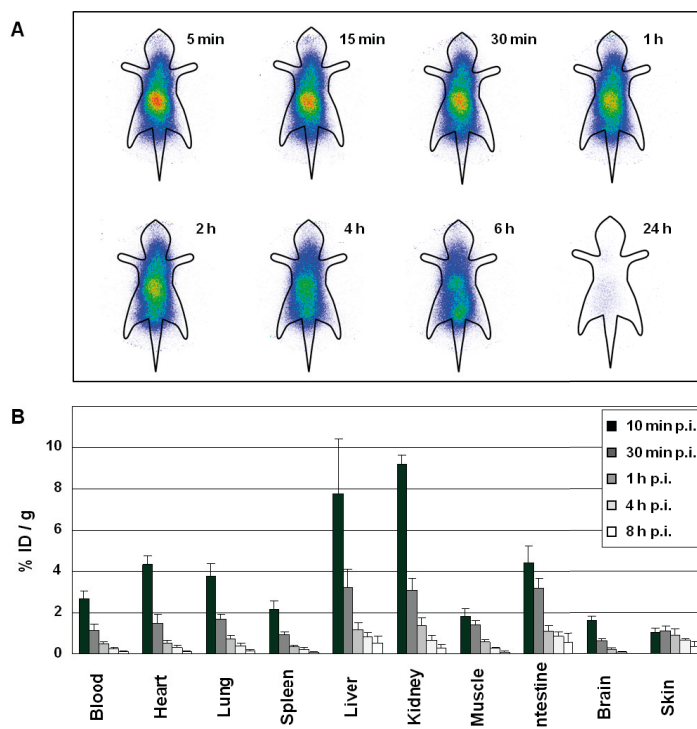


Figure 5. GF-15 is rapidly eliminated after intravenous injection. (A) Whole-body scintigraphic images of beige-nude-Xid mice at indicated times after intravenous

injection of the ^{125}I -labeled GF-15 analogue. (B) Biodistribution of a ^{131}I -labeled GF-15 analogue at different times after intravenous administration to beige-nude Xid mice ($n = 12$). Data are expressed as mean %ID/g \pm SD of each time point.

Figure 6

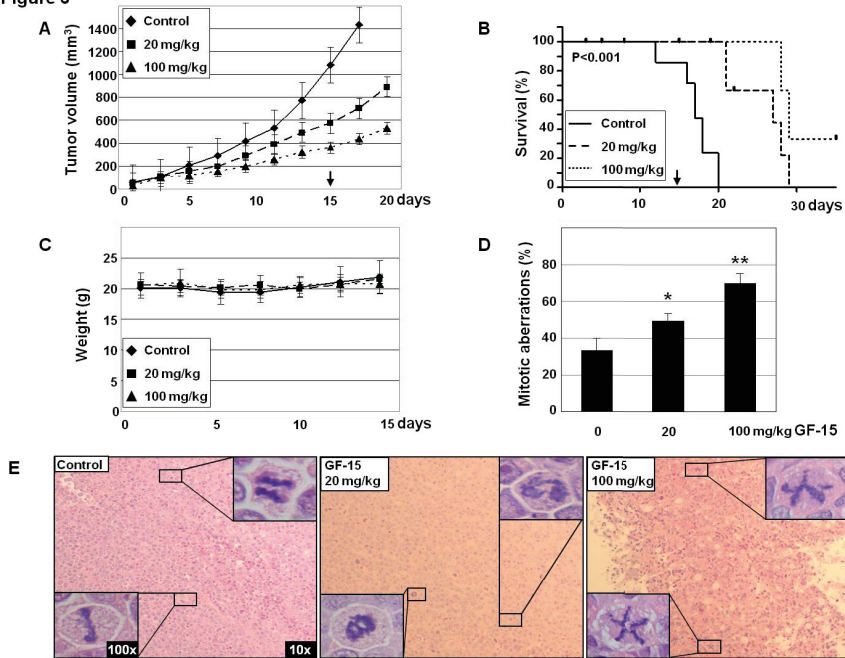
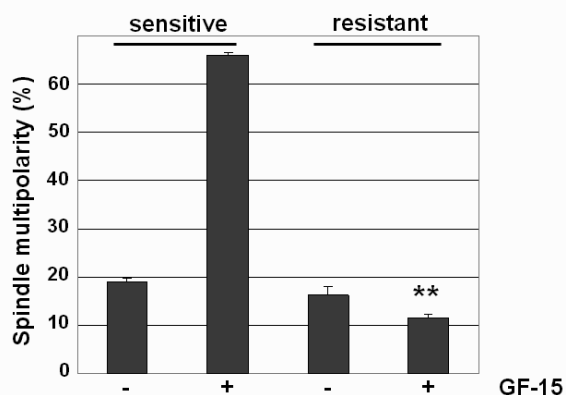


Figure 6. GF-15 induces mitotic aberrations, decreases tumor growth, and prolongs survival in a xenograft mouse model. Beige-nude Xid mice were subcutaneously inoculated in the right flank with 3×10^6 OPM2 cells. Treatment by intraperitoneal injection (vehicle alone or indicated concentrations) was started when tumors were measurable. Arrows indicates treatment stop. (A) Tumor burden was measured every alternating day using an electronic caliper. Tumor volume is presented as means \pm SE. (B) Survival was evaluated using Kaplan-Meier curves and log-rank analysis. (C) Body weight was evaluated three times per week. (D) In HE-stained tumor sections ($n = 3$ per cohort) at least 200 mitotic cells were counted

for mitotic aberrations. (E) Dose-dependent effects of GF-15 on mitotic figures. Paraffin-embedded sections of tumor tissue, explanted 24 hrs after last treatment, were HE-stained and analyzed by light microscopy. Representative microscopic images are shown.

Suppl. Fig. 1



Suppl. figure 1. GF-15 does not induce spindle multipolarity in resistant cells

SCC114 cells resistant to EC_{50} concentrations of GF15 after long-term culture under increasing concentrations of GF-15 do not display spindle multipolarity upon treatment with 5 μ M GF-15 for 24h. Centrosomes were counted in interphase by γ -tubulin staining.

Co-author Statements

In the following are the signed co-author statements from the three accepted publications (Chapters 3, 5 and 7) as well as the two manuscripts in Chapters 4 and 6.

January 2010

Joint author statement

If a thesis contains articles made in collaboration with other researchers, a joint author statement about the PhD-student's part of the article shall be made by each of the co-authors, cf. article 12, section 4 of the Ministerial Order No. 18 February 2008 about the PhD degree

Title of the article: Synthesis and Structure-Activity Relationship of Glucosylated Analogues as Inhibitors of Centrosomal Clustering in Cancer Cells

Author(s): Mads H. Rønnest, Blanka Rebacz, Lene Markworth, Anette H. Terp, Thomas O. Larsen, Alwin Krämer, Mads H. Clausen

Journal: Journal of Medicinal Chemistry

PhD-student: Mads H. Rønnest

CPR-no.: 050479-3443

Signature of the PhD-student:



Date: 8 December 2010

Co-author: Blanka Rebacz

Signature:



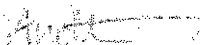
Co-author: Lene Markworth

Signature:



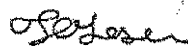
Co-author: Anette H. Terp

Signature:



Co-author: Thomas O. Larsen

Signature:



Co-author: Alwin Krämer

Signature:



Co-author: Mads H. Clausen

Signature:



*Technical University of Denmark**Research and Innovation, PhD programme**January 2010***Description of each author's contribution to the above-mentioned article:**

Mads H. Rønne: Ideas for as well as writing of the paper. Synthesis and/or purification of compounds 2, 4, 5, 6, 10, 11, 12, 16, 17, 18, 19, 20, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 34 and characterization of compounds 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 16, 17, 18, 19, 20, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35.

Blanka Rebacz: Initial screening of griseofulvin analogues as well as the test in triplicate used for the article. Writing of the paper.

Lene Markworth: Synthesis of compounds 2, 7, 8, 9, 13, 14, 15, 18, 19, 21, 23, 28, 29, 30, 32, 33, 34, 35 and characterization of compounds 2, 7, 8, 9, 13, 14, 15, 18, 19, 21, 23, 28, 29, 30, 32, 33, 34, 35.

Anette H. Terp: Initial screening of griseofulvin analogues

Thomas O. Larsen: Feedback and support on the fungal aspect of the paper.

Alwin Krämer: Feedback and support with the assay

Mads H. Clausen: Feedback and support on the syntheses present in the paper. Idea for as well as writing of the paper.

All co-authors have been proofreading the manuscript.

Joint author statements shall be delivered to the **PhD administration** together with the PhD thesis.

January 2010

Joint author statement

If a thesis contains articles made in collaboration with other researchers, a joint author statement about the PhD-student's part of the article shall be made by each of the co-authors, cf. article 12, section 4 of the Ministerial Order No. 18 February 2008 about the PhD degree

Title of the article: Disparate SAR Data of Griseofulvin Analogs for the Dermatophytes *Trichophyton mentagrophytes*, *T. rubrum* and MDA-MB-231 Cancer Cells

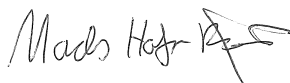
Author(s): Mads H. Rønnest, Marc S. Raab, Simon Anderhub, Sven Boesen, Alwin Krämer, Thomas O. Larsen, Mads H. Clausen

Journal: Journal of Medicinal Chemistry

PhD-student: Mads H. Rønnest

CPR- 050479-3443
no.:

Signature of the
PhD-student:



Date: 20 June 2011

Co-author: Marc S. Raab

Signature:



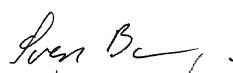
Co-author: Simon Anderhub

Signature:



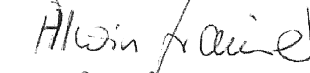
Co-author: Sven Boesen

Signature:



Co-author: Alwin Krämer

Signature:



Co-author: Thomas O. Larsen

Signature:



Co-author: Mads H. Clausen

Signature:



Technical University of Denmark

Research and Innovation, PhD programme

January 2010

Description of each author's contribution to the above-mentioned article:

Mads H. Rønne: Idea for as well as writing of the paper. Synthesis and characterization of all compounds in paper. Initial setup of the anti-fungal assay, finalizing the setup as well as testing all compounds in the anti-fungal assay. All processing of data and IC₅₀ calculations.

Marc S. Raab: Idea for as well as feedback and support on the work with the anti-cancer assay

Simon Anderhub: Feedback and support on the work with the anti-cancer assay

Sven Boesen: Initial setup of the anti-fungal assay.

Alwin Krämer: Idea for as well as feedback and support on the work with the anti-cancer assay

Thomas O. Larsen: Idea for as well as feedback and support on the work with the anti-fungal assay.

Mads H. Clausen: Idea for as well as feedback and support on the syntheses present in the paper and the anti-fungal assay.

All co-authors have been proofreading the manuscript.

Joint author statements shall be delivered to the **PhD administration** together with the PhD thesis.

January 2010

Joint author statement

If a thesis contains articles made in collaboration with other researchers, a joint author statement about the PhD-student's part of the article shall be made by each of the co-authors, cf. article 12, section 4 of the Ministerial Order No. 18 February 2008 about the PhD degree

Title of the article: Synthesis and single crystal X-ray analysis of two griseofulvin metabolites

Author(s): Mads H. Rønneest, Pernille Harris, Charlotte H. Gotfredsen, Thomas O. Larsen, Mads H. Clausen

Journal: Tetrahedron Letters

PhD-student: Mads H. Rønneest

CPR- 050479-3443
no.:

Signature of the
PhD-student:



Date: 27 October
2010

Co-author: Pernille Harris

Signature:



Co-author: Charlotte H. Gotfredsen

Signature:



Co-author: Thomas O. Larsen

Signature:



Co-author: Mads H. Clausen

Signature:



*Technical University of Denmark**Research and Innovation, PhD programme**January 2010***Description of each author's contribution to the above-mentioned article:**

Mads H. Rønnest: Synthesis of all compounds present in the paper, characterization of these, Idea for as well as writing the paper. Proofreading the manuscript.

Pernille Harris: All work concerning X-ray and analysis of data set. Proofreading the manuscript.

Charlotte H. Gotfredsen: Performed the 2D NMR experiments and gave general feedback and support on the NMR work present in the article. Proofreading the manuscript.

Thomas O. Larsen: Proofreading the manuscript

Mads H. Clausen: Feedback and support on the synthesis present in the paper and idea for as well as writing of the paper. Proofreading the manuscript.

Joint author statements shall be delivered to the **PhD administration** together with the PhD thesis.

January 2010

Joint author statement

If a thesis contains articles made in collaboration with other researchers, a joint author statement about the PhD-student's part of the article shall be made by each of the co-authors, cf. article 12, section 4 of the Ministerial Order No. 18 February 2008 about the PhD degree

Title of the article: A technique for selective *ipso*-substitution of *ortho*-, *meta*- or *para*-aryl trimethylsilyl groups with radioactive iodide in quantitative yields.

Author(s): Mads H. Rønneest, Felix Nissen, Thomas Ostenfeld Larsen, Walter Mier, Mads H. Clausen

Journal: To be decided

PhD-student: Mads H. Rønneest

CPR- 050479-3443
no.:

Signature of the
PhD-student:



Date: 15 June 2011

Co-author: Felix Nissen

Signature:



Co-author: Thomas Ostenfeld Larsen

Signature:



Co-author: Walter Mier

Signature:



Co-author: Mads H. Clausen

Signature:



*Technical University of Denmark**Research and Innovation, PhD programme**January 2010***Description of each author's contribution to the above-mentioned article:**

Mads H. Rønnest: Ideas for as well as writing of the paper. Development of the thallium/iodation mediated radiolabeling procedure. Synthesis and characterization of compounds. "Cold" synthesis of iodo griseofulvin analogs. Proofread the manuscript.

Felix Nissen: Development of the thallium/iodation mediated radiolabeling procedure. "Hot" syntheses of iodo griseofulvin analogs. Proofread the manuscript.

Thomas Ostenfeld Larsen: Proofread the manuscript.

Walter Mier: Development of the thallium/iodation mediated radiolabeling procedure. "Hot" syntheses of iodo griseofulvin analogs. Proofread the manuscript.

Mads H. Clausen: Feedback and support on the syntheses present in the paper. Idea for the project/research and the paper. Proofread the manuscript.

January 2010

Joint author statement

If a thesis contains articles made in collaboration with other researchers, a joint author statement about the PhD-student's part of the article shall be made by each of the co-authors, cf. article 12, section 4 of the Ministerial Order No. 18 February 2008 about the PhD degree

Title of the article: (+)-geodin from *Aspergillus terreus*

Author(s): Mads H. Rønne, Morten T. Nielsen, Blanka Leber, Uffe H. Mortensen, Alwin Krämer, Pernille Harris, Mads H. Clausen, Thomas O. Larsen

Journal: Acta Crystallographica Section C: Crystal Structure Communications

PhD-student: Mads H. Rønne

CPR- no.: 050479-3443

Signature of the PhD-student:



Date: 15 September 2010

Co-author: Morten T. Nielsen

Signature:



Co-author: Blanka Leber

Signature:



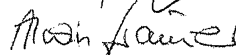
Co-author: Uffe H. Mortensen

Signature:



Co-author: Alwin Krämer

Signature:



Co-author: Pernille Harris

Signature:



Co-author: Mads H. Clausen

Signature:



Co-author: Thomas O. Larsen

Signature:



*Technical University of Denmark**Research and Innovation, PhD programme**January 2010***Description of each author's contribution to the above-mentioned article:**

Mads H. Rønne: Production of raw extract from *Aspergillus terreus*. Isolation of geodin from raw extract as well as characterization of geodin. Crystallization of crystal for X-ray analysis. The idea for as well as writing of the paper. Proofreading the manuscript.

Morten T. Nielsen: Production of raw extract from *Aspergillus terreus* and writing of the paper. Proofreading the manuscript.

Blanka Leber: Testing of geodin in cancer assay. Proofreading the manuscript.

Uffe H. Mortensen: Feedback and support on the production of raw extract from *Aspergillus terreus*. Proofreading the manuscript.

Alwin Krämer: Feedback and support on testing geodin. Proofreading the manuscript.

Pernille Harris: All work concerning X-ray data and structure analysis. Writing as well as proofreading the manuscript.

Mads H. Clausen: Feedback and support on characterization of geodin as well as idea for the paper. Proofreading the manuscript.

Thomas O. Larsen: Feedback and support on isolation of geodin as well as idea for the paper. Proofreading the manuscript.

Joint author statements shall be delivered to the **PhD administration** together with the PhD thesis.

Supporting Information - Chapter 3

Synthesis and Structure-Activity Relationship of Griseofulvin Analogues as Inhibitors of Centrosomal Clustering in Cancer Cells

Mads H. Rønneest, Blanka Rebacz, Lene Markworth, Anette H. Terp, Alwin Krämer, Thomas O. Larsen,

Mads H. Clausen

Supporting Information

S2 Combustion analysis (Table S1).

S3-9 Experimental

S10-S42 NMR spectra of **2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35**.

Table S1. Combustion analysisbb.

Compound	Formula	Anal. Calc'd	Anal. found
2	C ₁₆ H ₁₅ ClO ₆	C 56.73, H 4.46	56.75, 4.49
7	C ₁₇ H ₁₆ ClNO ₈	C 51.33, H 4.05	51.20, 3.98
10	C ₁₈ H ₁₉ ClO ₆	C 58.94, H 5.22	58.78, 5.20
11	C ₁₉ H ₂₁ ClO ₆	C 59.92, H 5.56	59.86, 5.47
12	C ₁₉ H ₂₁ ClO ₆	C 59.92, H 5.56	59.75, 5.60
13	C ₂₀ H ₂₃ ClO ₆	C 60.84, H 5.87	60.71, 5.83
14	C ₂₁ H ₂₅ ClO ₆	C 61.69, H 6.16	61.73, 5.99
15	C ₂₂ H ₂₇ ClO ₆	C 62.48, H 6.44	62.39, 6.37
16	C ₂₀ H ₂₁ ClO ₆	C 61.15, H 5.39	60.95, 5.45
18	C ₁₆ H ₁₄ Cl ₂ O ₅	C 53.80, H 3.95	53.69, 3.88
21	C ₂₃ H ₂₁ ClO ₅ S	C 62.09, H 4.76	62.15, 4.78
27	C ₁₉ H ₂₁ ClO ₆	C 57.23, H 5.93	57.27, 5.90
28	C ₁₇ H ₁₈ ClNO ₆	C 55.52, H 4.93	55.60, 4.96
35	C ₂₃ H ₂₀ ClIO ₆	C 49.80, H 3.63	49.73, 3.67

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(6'-methyl-cyclohex-2',4'-dione) 2.

To a solution of griseofulvin (7.0 g, 19.8 mmol) in HOAc (50 mL) was added 2 M H₂SO₄ (13 mL). The mixture was stirred at 80 °C for 45 min. where crystals of the product started to separate. The mixture was cooled to 20 °C, the product collected and then washed successively with cold EtOAc. The crystals were dried to afford griseofulvic acid **2**. Yield: 6.2 g (93%) (white crystals); *R*_f (MeOH:DCM 1:9): 0.31; m.p.: 262-263 °C (Litt.¹); IR(KBr, cm⁻¹): 3244, 1657, 1606 (Litt.²); ¹H NMR(500 MHz, DMSO-*d*₆): δ 11.90 (1H, s), 6.46 (1H, s), 5.31 (1H, s), 4.03 (3H, s), 3.91 (3H, s), 2.89-2.70 (2H, m), 2.52-2.43 (1H, m), 0.85 (3H, d, *J* = 6.2 Hz) (Litt.^{3,17}); ¹³C NMR(50 MHz, DMSO-*d*₆): δ 191.1, 187.7, 179.3, 170.0, 164.1, 157.4, 104.3, 101.4, 95.2, 94.3, 90.9, 57.5, 56.5, 34.4, 33.0, 14.2; Anal. [C₁₆H₁₅ClO₆]: C, H.

(2*S*,6'*R*)-(7-Chloro-6-methoxy-4-hydroxy-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-en-4'-one) 4.

A solution of 2 M MgI₂ was prepared by adding magnesium turnings (0.88 g, 36.0 mmol) and iodine (4.49 g, 17.7 mmol) to anhydrous Et₂O (6 mL) and toluene (12 mL). This solution was refluxed at 80 °C for 2 h, filtered and added to a solution of griseofulvin (3.03 g, 8.5 mmol) in anhydrous toluene (20 mL) at 80 °C. The solution was cooled to 20 °C after 3 h. The mixture was diluted with EtOAc (50 mL) and washed with sat. aq. NH₄Cl (40 mL) and then 10% aq. Na₂S₂O₃ (40 mL). The combined aqueous phases were extracted with EtOAc (3×60 mL) and the organic layer was dried (MgSO₄) and concentrated. The crude mixture was purified by reverse phase chromatography (C-18, MeOH and water gradient) yielding **4**. Yield: 521 mg (18%) (yellow crystals); *R*_f (MeOH:CH₂Cl₂ 1:9, 1% HOAc): 0.45; IR(KBr, cm): 1627, 1559; ¹H NMR(500 MHz, DMSO-*d*₆): δ 7.68 (1H, s), 6.49 (1H, s), 5.58 (1H, s), 3.89 (3H, s), 3.62 (3H, s), 2.77 (1H, ddq, *J* = 12.6, 6.5, 4.8 Hz), 2.67 (1H, dd, *J* = 16.3, 13.4 Hz), 2.33 (1H, dd, *J* = 16.3, 4.5 Hz), 0.80 (3H, d, *J* = 6.5 Hz) ¹³C NMR(50 MHz, DMSO-*d*₆): δ 195.9, 193.8, 171.1, 170.9, 166.5, 165.8, 106.6, 104.5, 97.3, 89.8 (2C), 56.9, 56.5, 39.4, 35.9, 13.8; HRMS (ESI+) calcd for [C₁₆H₁₆ClO₆]⁺ 339.0635, found 339.0633.

General procedure for the synthesis of ring A ethers **5** and **6**.

To a solution of **4** (0.7 mmol, 1 equiv.) in DMF (3 mL, 0.2 M) was added NaH (0.7 mmol, 1 equiv.) and tetrabutylammonium iodide (0.1 mmol, 0.1 equiv.). The mixture was stirred at 100 °C and after 20 min. The alkyl bromide (1.1 mmol, 1.5 equiv.) was added. After 24 h the solution was diluted with EtOAc (20 mL) and washed with sat. aq. NH₄Cl (20 mL). The aqueous phase was extracted with EtOAc (3×30 mL), the combined organic phases were dried (MgSO₄) and concentrated. The residue was purified by column chromatography (EtOAc:heptane 1:2) affording the desired product, which was re-crystallized from CH₂Cl₂/heptane.

(2*S*,6'*R*)-(7-Chloro-4-ethoxy-6-methoxy-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-en-4'-one) 5.

Yield: 39 mg (15%) (white crystals); *R*_f (EtOAc:heptane 5:1): 0.55; m.p.: 205-206 °C (Litt.¹); ¹H NMR(500 MHz, CDCl₃): δ 6.12 (1H, s), 5.53 (1H, s), 4.25-4.15 (2H, m), 4.00 (3H, s), 3.61 (3H, s), 3.04 (1H, dd, *J* = 16.8, 13.5 Hz), 2.84 (1H, ddq, *J* = 13.5, 4.7, 6.7 Hz), 2.42 (1H, dd, *J* = 16.8, 4.7 Hz), 1.52 (3H, t, *J* = 7.0 Hz), 0.96 (3H, d, *J* = 6.7 Hz) (Litt.⁴); ¹³C NMR(50 MHz, CDCl₃): δ 196.9, 192.2, 170.8, 169.5, 164.4, 157.2, 105.6, 104.7, 97.0, 90.6, 90.3, 65.1, 56.9, 56.6, 40.0, 36.4, 14.3, 14.2; HRMS (ESI+) calcd for [C₁₈H₂₀ClO₆]⁺ 367.0948, found 367.0944.

(2*S*,6'*R*)-(7-Chloro-4-benzoyloxy-6-methoxy-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-en-4'-one) 6.

Yield: 40 mg (14%) (white crystals); *R*_f (EtOAc:heptane 4:1): 0.69; m.p.: 237-239 °C (Litt.¹); ¹H NMR(500 MHz, CDCl₃): δ 7.49-7.46 (2H, m), 7.42-7.38 (2H, m), 7.36-7.31 (1H, m), 6.17 (1H, s), 5.55 (1H, s), 5.27 (2H, s), 3.94 (3H, s), 3.63 (3H, s), 3.05 (1H, dd, *J* = 16.6, 13.6 Hz), 2.85 (1H, m), 2.44 (1H, dd, *J* = 16.6, 4.6 Hz), 0.98 (3H, d, *J* = 6.7 Hz); ¹³C NMR(50 MHz, CDCl₃): δ 196.9, 192.1, 170.8, 169.4, 164.3, 156.6, 135.4, 128.8 (2C), 128.3, 126.8 (2C), 104.8 (2C), 97.4, 91.5, 90.7, 71.1, 56.9, 56.6, 40.0, 36.5, 14.4; HRMS (ESI+) calcd for [C₂₃H₂₂ClO₆]⁺ 429.1105, found 429.1108.

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-5-nitro-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-ene-4'-one) 7.

To a solution of griseofulvin (2.0 g, 5.67 mmol) in acetic anhydride (10 mL) was slowly added a solution of nitric acid (2 mL) in acetic anhydride (4 mL) while maintaining the temperature at 0-5 °C. The mixture was stirred at 20 °C for 48 hours and then cooled to 0 °C. A 20% HCl solution was added cautiously to the mixture until a yellow precipitate began to appear, cooling was continued to complete the precipitation of the product and the product was collected by filtration. Additional product was obtained by cautiously adding water to the mother liquor and collected by filtration. The residue was purified by column chromatography (EtOAc:heptane 1:2) to afford the product **7** which was re-crystallized from EtOAc. Yield: 744 mg (33%) (yellow needles); R_f (EtOAc:heptane 5:1): 0.76; m.p.: 195-197 °C (Litt.³); IR (KBr, cm^{-1}): 1719, 1669, 1617, 1577 (Litt.³); ^1H NMR (300 MHz, CDCl_3): δ 5.59 (1H, s), δ 4.22 (3H, s), δ 4.14 (3H, s), δ 3.67 (3H, s), δ 2.99-2.95 (1H, m), δ 2.90-2.86 (1H, m), δ 2.49 (1H, dd, $J = 14.5, 2.4$ Hz), δ 0.98 (3H, d, $J = 6.4$ Hz) (Litt.¹⁷); ^{13}C NMR (75 MHz, CDCl_3): δ 196.3, 192.5, 170.0, 169.6, 169.3, 157.4, 149.5, 105.2, 108.9, 91.2, 91.1, 57.2, 64.5, 63.3, 40.0, 36.6, 14.5; EIMS [$\text{C}_{17}\text{H}_{16}\text{ClNO}_8$] 397, found 397; Anal. ($\text{C}_{17}\text{H}_{16}\text{ClNO}_8$) C, H.

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-5-amino-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-ene-4'-one) **8**.

Powdered iron (1.458 g, 26.11 mmol) was added over 3 h to a stirred solution of 5-nitro griseofulvin **7** (744 mg, 1.86 mmol) in 90% aq. acetic acid (18.6 mL). The mixture was refluxed for 1 h and cooled to 20 °C where CH_2Cl_2 (12.6 mL) and then sat. aq. NaHCO_3 was added until pH 7. The aqueous phase was extracted with CH_2Cl_2 (4×100 mL). The combined organic phases were dried (Na_2SO_4) and concentrated. The residue was purified by column chromatography (EtOAc:heptane 1:1) to afford the product **8** which was re-crystallized from EtOAc/heptane. Yield: 369 mg (58%) (yellow crystals); R_f (EtOAc:heptane 5:2): 0.46; m.p.: 120-123 °C (Litt.³); IR (KBr, cm^{-1}): 3471, 3376, 1706, 1664 (Litt.³); ^1H NMR (CDCl_3): δ 5.53 (1H, s), 4.06 (3H, s), 3.98 (3H, s), 3.84 (2H, s), 3.61 (3H, s), 2.95 (1H, dd, $J = 16.2, 13.3$ Hz), 2.87-2.75 (1H, m), 2.41 (1H, dd, $J = 16.2, 4.2$ Hz), 0.91 (3H, d, $J = 6.6$ Hz) (Litt.³); ^{13}C NMR (CDCl_3): δ 196.8, 194.4, 171.3, 162.1, 153.0, 142.5, 129.5, 110.0, 105.5, 105.2, 89.8, 62.5, 60.5, 56.9, 40.2, 36.8, 14.6; EIMS [$\text{C}_{17}\text{H}_{18}\text{ClNO}_6$] 367, found 367.

General procedure for the synthesis of enol ethers by solvolysis (**10-15**).

CSA (0.1 mmol, 0.1 equiv.) was added to a solution of griseofulvic acid (0.6 mmol, 1 equiv.) in the appropriate alcohol (6 mL, 0.1 M). The mixture was stirred at 100 °C for 6 hours, and then cooled to 20 °C. EtOAc (20 mL) was added to the solution and the mixture was washed with sat. NaH_2PO_4 (20 mL) and then water (20 mL). The combined aqueous phases were extracted with EtOAc (3×20 mL), dried (MgSO_4) and then concentrated. The residue was purified by column chromatography (toluene: CH_2Cl_2 :EtOAc 7:7:1) to afford the desired product and the isomer. When possible the product was re-crystallized from EtOAc/Heptane.

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(2'-ethoxy-6'-methyl-cyclohex-2'-en-4'-on) **10**.

Yield: 32 mg (14%) (white crystals); R_f (EtOAc:heptane 5:1): 0.59; m.p.: 202-204 °C (Litt.⁵); IR (KBr, cm^{-1}): 1708, 1661, 1614 (Litt.⁶); ^1H NMR (500 MHz, CDCl_3): δ 6.12 (1H, s), 5.51 (1H, s), 4.03 (3H, s), 3.98 (3H, s), 3.93-3.74 (2H, m), 3.02 (1H, dd, $J = 16.7, 13.4$ Hz), 2.91-2.76 (1H, m), 2.42 (1H, dd, $J = 16.7, 4.7$ Hz), 1.21 (3H, t, $J = 7.0$ Hz), 0.96 (3H, d, $J = 6.7$ Hz) (Litt.⁴); ^{13}C NMR (50 MHz, CDCl_3): δ 197.1, 192.6, 169.9, 169.6, 164.4, 157.6, 105.1 (2C), 97.1, 90.8, 89.3, 65.3, 56.9, 56.3, 39.9, 36.3, 14.2, 13.6; HRMS (ESI+) calcd for [$\text{C}_{18}\text{H}_{20}\text{ClO}_6$]⁺ 367.0949, found 367.0960; Anal. ($\text{C}_{18}\text{H}_{19}\text{ClO}_6$) C, H.

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(6'-methyl-2'-propoxy-cyclohex-2'-en-4'-on) **11**.

Yield: 41 mg (18%) (white crystals); R_f (EtOAc:heptane 5:1): 0.64; m.p.: 151-153 °C (Litt.⁷) IR (KBr, cm^{-1}): 1706, 1662, 1614 (Litt.⁶); ^1H NMR (500 MHz, CDCl_3): δ 6.13 (1H, s), 5.51 (1H, s), 4.04 (3H, s), 3.98 (3H, s), 3.79-3.66 (2H, m), 3.05 (1H, dd, $J = 16.7, 13.5$ Hz), 2.89-2.80 (1H, m), 2.42 (1H, dd, $J = 16.7, 4.7$ Hz), 1.64-1.55 (2H, m), 0.97 (3H, d, $J = 6.7$ Hz), 0.82 (3H, t, $J = 7.4$ Hz); ^{13}C NMR (50 MHz, CDCl_3): δ 197.1, 192.6, 170.0 (2C), 164.4, 157.6, 105.1, 104.8, 96.8, 90.8, 89.3, 70.7, 56.9, 56.3, 39.9, 36.2, 21.5, 14.2, 9.9; HRMS (ESI+) calcd for [$\text{C}_{19}\text{H}_{22}\text{ClO}_6$]⁺ 381.1105, found 381.1105; Anal. ($\text{C}_{19}\text{H}_{21}\text{ClO}_6$) C, H.

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(2'-isopropoxy-6'-methyl-cyclohex-2'-ene-4'-on) **12**.

Yield: 50 mg (22%) (off-white crystals); R_f (EtOAc:heptane 5:1): 0.62; m.p.: 194-197 °C (Litt.¹); IR (KBr, cm^{-1}): 1714, 1667, 1615 (Litt.⁶); ^1H NMR (500 MHz, CDCl_3): δ 6.12 (1H, s), 5.51 (1H, s), 4.33 (1H, sept., J = 6.1 Hz), 4.03 (3H, s), 3.98 (3H, s), 3.02 (1H, dd, J = 16.7, 13.4 Hz), 2.87-2.77 (1H, m), 2.41 (1H, dd, J = 16.7, 4.7 Hz), 1.23 (3H, d, J = 6.1 Hz), 1.11 (3H, d, J = 6.1 Hz), 0.95 (3H, d, J = 6.7 Hz); ^{13}C NMR (50 MHz, CDCl_3): δ 197.2, 192.6, 169.6, 169.0, 164.3, 157.5, 105.4 (2C), 97.0, 90.9, 89.2, 72.4, 56.9, 56.3, 39.8, 36.2, 21.2, 20.7, 14.3; HRMS (ESI+) calcd for $[\text{C}_{19}\text{H}_{22}\text{ClO}_6]^+$ 381.1105, found 381.1119; Anal. ($\text{C}_{19}\text{H}_{21}\text{ClO}_6$) C, H.

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-butoxy-6'-methyl-cyclohex-2'-ene-4'-one) **13**.

Yield: 72 mg (14%) (white crystals); R_f (EtOAc:heptane 5:1): 0.41; m.p.: 152-154 °C (Litt.⁷); IR (KBr, cm^{-1}): 1706, 1662, 1614; ^1H NMR (300 MHz, CDCl_3): δ 6.11 (1H, s), δ 5.50 (1H, s), δ 4.03 (3H, s), δ 3.97 (3H, s), δ 3.83-3.68 (2H, m), δ 3.03 (1H, dd, J = 16.5, 13.5 Hz), δ 2.79 (1H, ddq, J = 13.3, 6.6, 4.7 Hz), δ 2.41 (1H, dd), δ 1.59-1.49 (2H, m), δ 1.28-1.19 (2H, m), δ 0.95 (3H, d, J = 6.7 Hz), δ 0.74 (3H, t, J = 7.4 Hz); ^{13}C NMR (75 MHz, CDCl_3): δ 197.1, 192.9, 170.4, 169.5, 164.7, 157.9, 105.4, 105.2, 97.2, 91.1, 89.6, 69.5, 57.2, 56.6, 40.2, 36.5, 30.2, 19.0, 14.5, 13.8; HRMS (ESI+) calcd for $[\text{C}_{20}\text{H}_{24}\text{ClO}_6]^+$ 395.1261, found 395.1263; Anal. ($\text{C}_{20}\text{H}_{23}\text{ClO}_6$) C, H.

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(6'-methyl-2'-pentoxy-cyclohex-2'-ene-4'-one) **14**.

Yield: 392 mg (17%) (white crystals); R_f (EtOAc:heptane 5:1): 0.52; m.p.: 146-148 °C (Litt.⁷); IR (KBr, cm^{-1}): 1701, 1653, 1613; ^1H NMR (300 MHz, CDCl_3): δ 6.11 (1H, s), 5.48 (1H, s), 4.02 (3H, s), 3.96 (3H, s), 3.79-3.66 (2H, m), 3.03 (1H, dd, J = 16.5, 13.5 Hz), 2.82 (1H, ddq, J = 13.5, 6.8, 4.7 Hz), 2.40 (1H, dd, J = 16.5, 4.6 Hz), 1.61-1.51 (2H, m), 1.20-1.15 (4H, m), 0.95 (3H, d, J = 6.7 Hz), 0.77 (3H, t, J = 6.8 Hz); ^{13}C NMR (75 MHz, CDCl_3): δ 197.2, 192.8, 170.4, 169.9, 164.7, 157.9, 105.4, 105.1, 97.4, 91.1, 89.5, 69.8, 57.2, 56.6, 40.2, 36.4, 28.0, 27.9, 22.3, 14.5, 14.1; HRMS (ESI+) calcd for $[\text{C}_{21}\text{H}_{26}\text{ClO}_6]^+$ 409.1418, found 409.1412; Anal. ($\text{C}_{21}\text{H}_{25}\text{ClO}_6$) C, H.

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-hexoxy-6'-methyl-cyclohex-2'-ene-4'-one) **15**.

Yield: 450 mg (19%) (white crystals); R_f (EtOAc:heptane 5:1): 0.52; m.p.: 152-154 °C (Litt.⁷); IR (KBr, cm^{-1}): 1701, 1663, 1616; ^1H NMR (300 MHz, CDCl_3): δ 6.11 (1H, s), 5.50 (1H, s), 4.03 (3H, s), 3.97 (3H, s), 3.83-3.66 (2H, m), 3.04 (1H, dd, J = 16.5, 13.5 Hz), 2.83 (1H, ddq, J = 13.3, 6.6, 4.6 Hz), 2.41 (1H, dd, J = 16.5, 4.6 Hz), 1.61-1.49 (2H, m), 1.25-1.16 (6H, m), 0.96 (3H, d, J = 6.7 Hz), 0.80 (3H, t, J = 6.9 Hz); ^{13}C NMR (75 MHz, CDCl_3): δ 197.2, 192.8, 170.4, 169.8, 164.7, 157.9, 105.5, 105.1, 97.6, 91.1, 89.5, 69.7, 57.2, 56.6, 40.2, 36.4, 31.3, 28.2, 25.4, 22.6, 14.5, 14.0; HRMS (ESI+) calcd for $[\text{C}_{22}\text{H}_{28}\text{ClO}_6]^+$ 423.1574, found 423.1560; Anal. ($\text{C}_{22}\text{H}_{27}\text{ClO}_6$) C, H.

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-chloro-6'-methyl-cyclohex-2'-ene-4'-one) **18**.

Griseofulvic acid (11.3 g, 31.6 mmol) was mixed with LiCl (4.2 g, 0.101 mol) and added to a solution of POCl_3 (15.6 mL, 0.168 mol) in dioxane (40 mL). The mixture was stirred at 100 °C for 30 min, cooled to 0 °C and sat. aq. Na_2CO_3 was added carefully to the solution until slightly basic (pH 7-8). The aqueous phase was extracted with CH_2Cl_2 (4×300 mL) and the combined organic phases were dried (MgSO_4) and concentrated. The residue was purified by column chromatography (toluene: CH_2Cl_2 :EtOAc 35:35:1) affording the desired product **18** and the isomer. The product was re-crystallized from CH_2Cl_2 /heptane. Yield: 234 mg (44%) (white crystals); R_f (EtOAc:heptane 5:1): 0.51; m.p.: 196-198 °C (Litt.⁸); IR (KBr, cm^{-1}): 1698, 1615 (Litt.⁸); ^1H NMR (300 MHz, CDCl_3): δ 6.41 (1H, s), 6.14 (1H, s), 4.03 (3H, s), 3.98 (3H, s), 3.12 (1H, dd, J = 16.7, 13.9 Hz), 2.89 (1H, ddq, J = 13.5, 4.4, 6.7 Hz), 2.45 (1H, dd, J = 16.7, 4.4 Hz), 0.98 (3H, J = 6.7 Hz); ^{13}C NMR (50 MHz, CDCl_3): δ 195.0, 191.1, 169.3, 165.3, 158.1, 152.9, 131.6, 105.4,

97.6, 91.7, 90.0, 57.3, 56.7, 40.3, 37.8, 15.2; EIMS calcd for $[C_{16}H_{14}Cl_2O_5]$ 356, found 356; Anal. ($C_{16}H_{14}Cl_2O_5$) C, H.

General procedure for the synthesis of enol ethers by addition-elimination. (**19-21**).

To a solution of **18** (0.65 mmol, 1 equiv.) in 1,4-dioxane (3 mL, 0.2 M) was added the desired alcohol (1.30 mmol, 2 equiv.) and DBU (1.63 mmol, 2.5 equiv.). The mixture was heated to 100 °C and stirred for 12 h. The mixture was then cooled to 20 °C and excess reagent was quenched with sat. aq. NH_4Cl (30 mL). The aqueous phase was extracted with EtOAc (3×30 mL) and the combined organic phases were dried ($MgSO_4$) and then concentrated. The residue was purified by column chromatography (heptane:EtOAc 3:2) affording the product. When possible the product was re-crystallized from EtOAc/heptane.

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(6'-methyl-2'-phenoxy-cyclohex-2'-ene-4'-one) **19**.

Yield: 199 mg (86%); R_f (EtOAc:heptane 5:1): 0.50; IR (KBr, cm^{-1}): 1704, 1665; 1H NMR(300 MHz, $CDCl_3$): δ 7.36-7.29 (2H, m), 7.22-7.16 (1H, m), 7.00-6.95 (2H, m), 6.13 (1H, s), 5.30 (1H, s), 4.01 (3H, s), 3.99 (3H, s) 3.06 (1H, dd, $J = 16.0, 13.5$ Hz), 2.99-2.85 (1H, m), 2.42 (1H, dd, $J = 16.0, 4.0$ Hz), 1.01 (3H, d, $J = 6.5$ Hz); ^{13}C NMR(75 MHz, $CDCl_3$): 197.0, 192.3, 170.9, 169.6, 164.8, 157.8, 152.6, 130.0 (2C), 126.3, 121.1 (2C), 108.8, 105.2, 97.2, 90.6, 89.5, 57.0, 56.4, 40.3, 36.5, 14.3; HRMS (ESI+) calcd for $[C_{22}H_{20}ClO_6]^+$ 415.0948, found 415.0941.

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(2'-benzyloxy-6'-methyl-cyclohex-2'-en-4'-one) **20**.

Yield: 30 mg (55%) (white crystals); R_f (EtOAc:heptane 5:1): 0.49; m.p.: 186-188 °C (Litt.¹); 1H NMR(300 MHz, $CDCl_3$): δ 7.32-7.24 (3H, m), 7.20-7.16 (2H, m), 6.11 (1H, s), 5.60 (1H, s), 4.02 (3H, s), 3.96 (3H, s), 4.92 (1H, d, $J = 12.3$ Hz), 4.82 (1H, d, $J = 12.3$ Hz), 3.06 (1H, dd, $J = 16.7, 13.4$ Hz), 2.87 (1H, ddq, $J = 13.4, 6.7, 4.8$ Hz), 2.44 (1H, dd, $J = 16.7, 4.8$ Hz), 0.99 (3H, d, $J = 6.7$ Hz); ^{13}C NMR(50 MHz, $CDCl_3$): δ 196.9, 192.3, 169.5 (2C), 164.5, 157.7, 134.6, 128.5 (2C), 128.1, 126.6 (2C), 105.9, 105.2, 97.2, 90.7, 89.4, 70.6, 56.9, 56.3, 40.0, 36.6, 14.2; HRMS (ESI+) calcd for $[C_{23}H_{22}ClO_6]^+$ 429.1105, found 429.1103.

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-benzylthio-6'-methyl-cyclohex-2'-ene-4'-one) **21**.

Yield: 349 mg (93%) (white crystals); R_f (EtOAc:heptane 5:1): 0.44; m.p.: 208-210 °C (Litt.⁸); IR (KBr, cm^{-1}): 1703, 1663 (Litt.⁸); 1H NMR (300 MHz, $DMSO-d_6$): δ 7.33-7.27 (5H, m), 6.51 (1H, s), 6.09 (1H, s), 4.17 (1H, d, $J = 12.5$ Hz), 4.05 (1H, d, $J = 12.5$ Hz), 4.03 (3H, s), 3.95 (3H, s), 2.95-2.83 (1H, m), 2.68 (1H, dd, $J = 17.3, 13.3$ Hz), 2.40 (1H, dd, $J = 17.2, 5.3$ Hz), 0.77 (3H, d, $J = 6.6$ Hz); ^{13}C NMR (75 MHz, $DMSO-d_6$): δ 193.4, 191.1, 168.5, 165.2, 160.5, 158.4, 135.3, 129.8 (2C), 129.4 (2C), 128.4, 122.6, 104.1, 95.8, 92.3, 92.2, 58.3, 57.3, 49.9, 37.4, 35.4, 14.9; HRMS (ESI+) calcd for $[C_{23}H_{22}ClO_5S]^+$ 445.0877, found 445.0872; Anal. ($C_{23}H_{21}ClO_5S$) C, H.

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-en-4'-ol) **26**.

A solution of griseofulvin (212 mg, 0.6 mmol) in MeOH (3 mL) was cooled to -40 °C. $CeCl_3$ (448 mg, 1.8 mmol) was added and the solution stirred for 10 min. after which $NaBH_4$ (70 mg, 1.8 mmol) was added. After 3 h excess reagent was quenched by the slow addition of acetone (3 mL) and the mixture was allowed to reach 20 °C, diluted with EtOAc (10 mL) and washed with water (20 mL). The aqueous phase was extracted with EtOAc (5×30 mL) and the combined organic phases were dried ($MgSO_4$) and concentrated. The compound **26** was re-crystallized from MeOH. Yield: 209 mg (98%) (white crystals); R_f (EtOAc:heptane 5:1): 0.25; m.p.: 110-113 °C. (Litt.⁹); 1H NMR(300 MHz, $CDCl_3$): δ 6.08 (1H, s), 5.11 (1H, d, $J = 1.9$ Hz), 4.45 (1H, ddd, $J = 9.3, 5.8, 1.9$ Hz), 4.00 (3H, s), 3.96 (3H, s), 3.44 (3H, s), 2.37 (1H, ddq, $J = 13.1, 3.4, 6.8$ Hz), 2.10 (1H, dt, $J = 9.3, 13.1$ Hz), 2.00 (1H, dddd, $J = 13.1, 5.8, 3.4, 0.9$ Hz), 0.87 (3H, d, $J = 6.8$ Hz); ^{13}C NMR (from gHSQC, gHMBC, 500 MHz, $CDCl_3$): δ 195.5, 169.6, 164.8, 157.0, 152.6, 105.8, 105.5, 96.9, 92.0, 88.8, 65.9, 56.5, 56.1, 55.0, 36.3, 35.6, 14.2; EIMS $[C_{17}H_{19}ClO_6] M^+$ 354, found 354.

(2*S*,2'*S*,4'*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohexan-4'-ol) **27**.

To a solution of griseofulvin (107 mg, 0.3 mmol) in MeOH (3 mL) was added 5% Pt on charcoal (24 mg). The mixture was stirred at 20 °C for 12 h under a hydrogen atmosphere. EtOAc (20 mL) was added and the mixture was filtered, dried (MgSO₄), concentrated and purified by column chromatography (EtOAc:heptane 1:1). The product **27** was re-crystallized from EtOAc/heptane. Yield: 94 mg (86%) (white crystals); R_f (EtOAc:heptane 4:1): 0.27; m.p.: 188-190 °C (Litt.¹⁰); IR(KBr, cm⁻¹): 1696; ¹H NMR(500 MHz, CDCl₃): δ 6.06 (1H, s), 3.99 (3H, s), 3.94 (3H, s), 3.83-3.70 (1H, m), 3.62 (1H, dd, *J* = 12.0, 5.1 Hz), 3.27 (3H, s), 2.29 (1H, m), 2.24 (1H, q, *J* = 12.0 Hz), 2.09 (2H, m), 1.90-1.78 (1H, m), 0.82 (3H, d, *J* = 6.1 Hz); ¹³C NMR(50 MHz, CDCl₃): δ 195.8, 169.0, 163.6, 156.9, 107.7, 96.6, 94.8, 88.6, 81.5, 67.4, 58.4, 56.7, 56.0, 37.4, 35.0, 34.9, 14.1; Anal. (C₁₇H₂₁ClO₆) C, H.

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-ene-4'-one-4'-oxime) **28**.

To a solution of **1** (0.20 mmol, 1.0 equiv.) in EtOH (5 mL, 0.03M) and DMSO (2.5 mL, 0.03M) was added hydroxylamine hydrochloride (0.70 mmol, 3.5 equiv.) and sodium acetate (0.86 mmol, 4.3 equiv.). The mixture was stirred at 75 °C for 24 hours, allowed to reach 20 °C and diluted with CH₂Cl₂ (20 mL). The mixture was washed with distilled water (2×15 mL) and then brine (15 mL). The organic phase was dried (MgSO₄) and concentrated. The crude mixture was purified by column chromatography (toluene:CH₂Cl₂:EtOAc 2:2:1) to afford the desired product.

Yield: 988 mg (95%) (white needles); R_f (MeOH:CH₂Cl₂ 1:10): 0.50; IR (KBr, cm⁻¹): 1706, 1590, 1614 (Litt.³); ¹H NMR (300 MHz, CDCl₃): δ 6.26 (0.5H, s), 6.10 (0.5H, s), 6.10 (0.5H, s), 5.59 (0.5H, s), 4.01 (3H, s), 3.96 (1.5H, s), 3.96 (1.5H, s), 3.61 (1.5H, s), 3.55 (1.5H, s), 3.12 (0.5H, dd, *J* = 16.6, 4.7 Hz), 2.99 (0.5H, dd, *J* = 15.0, 13.1 Hz), 2.70 (0.5H, dd, *J* = 16.6, 13.0 Hz), 2.64-2.48 (1H, m), 2.40 (0.5H, dd, *J* = 15.0, 4.2 Hz), 0.95 (1.5H, d, *J* = 6.7 Hz), 0.94 (1.5H, d, *J* = 6.7 Hz) (Litt.³); ¹³C NMR (75 MHz, CDCl₃): δ 194.1 (0.5C), 193.9 (0.5C), 169.4 (0.5C), 169.4 (0.5C), 164.3 (0.5C), 164.2 (0.5C), 161.3 (0.5C), 158.6 (0.5C), 157.5 (0.5C), 157.4 (0.5C), 155.0 (0.5C), 151.8 (0.5C), 105.6 (0.5C), 105.5 (0.5C), 98.9 (0.5C), 97.1, 92.6 (0.5C), 91.4 (0.5C), 91.3 (0.5C), 89.1, 56.9 (0.5C), 56.9 (0.5C), 56.3, 56.1 (0.5C), 55.9 (0.5C), 36.4 (0.5C), 35.2 (0.5C), 30.8 (0.5C), 25.5 (0.5C), 14.3 (0.5C), 14.2 (0.5C); HRMS (ESI⁺) calcd for [C₁₇H₁₉ClNO₆]⁺ 368.0901, found 368.0891; Anal. (C₁₇H₁₈ClNO₆) C, H.

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(3',3',6'-trimethyl-cyclohex-2',4'-dione) **31**.

To a solution of griseofulvin (514 mg, 1.5 mmol) in THF (10 mL) was added K₂CO₃ (410 mg, 3.0 mmol) and MeI (0.2 mL, 3.1 mmol) and the mixture was heated to 80 °C. The solution was cooled to 20 °C after 12 h. The reaction was quenched with sat. aq. NH₄Cl (30 mL) and the aqueous phase was extracted with EtOAc (3×30 mL). The combined organic phases were dried (MgSO₄), concentrated and the residue was purified by column chromatography (EtOAc:heptane 1:6) affording the product **31**. Yield: 75 mg (14%) (yellow oil); R_f (EtOAc:heptane 5:1): 0.65; IR(KBr, cm⁻¹): 1695, 1615; ¹H NMR(500 MHz, CDCl₃): δ 6.11 (1H, s), 4.02 (3H, s), 3.94 (3H, s), 3.44-3.39 (1H, m), 2.88-2.77 (2H, m), 1.39 (3H, s), 1.36 (3H, s), 1.05 (3H, d, *J* = 6.5 Hz); ¹³C NMR(75 MHz, CDCl₃): δ 207.2, 201.6, 190.8, 169.2, 164.8, 157.9, 104.5, 97.3, 96.0, 89.6, 60.2, 57.0, 56.3, 40.4, 32.2, 23.6, 23.5, 14.6; EIMS [C₁₈H₁₉ClO₆] 366, found 366.

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(3'-benzyl-6'-methyl-cyclohex-2',4'-dione) **32**.

A solution of griseofulvic acid (5.0 g, 0.015 mol) in H₂O (7.4 mL) containing KOH (0.832 g, 0.015 mol) and NaI (2.22 g, 0.015 mol) was heated to reflux with copper powder (188 mg, 2.97 mmol) and benzyl bromide (2.54 g, 0.015 mol) for 24 hours. The mixture was cooled to 20 °C and the precipitate was filtered off. The filtrate was extracted with EtOAc (3×10 mL) and the organic phase was washed with sat. aq. NaHCO₃. The aqueous phase was acidified with 2M HCl and then extracted with EtOAc (3×50 mL). The combined organic phases were dried (MgSO₄) and concentrated. The residue was purified by column chromatography (toluene:CH₂Cl₂:EtOAc 10:10:1) to yield the product **32** which was re-crystallized from CH₂Cl₂/heptane.

Yield: 533 mg (8%) (white crystals); R_f (EtOAc:heptane 5:1): 0.33; m.p.: 202–204 °C (Litt.¹¹); IR (KBr, cm^{-1}): 1702, 1616 (Litt.¹¹); ^1H NMR (300 MHz, $\text{DMSO}-d_6$): δ 11.6 (1H, s), 7.21–7.16 (2H, m), 7.10–7.05 (3H, m), 6.43 (1H, s), 4.01 (3H, s), 3.88 (3H, s), 3.50 (1H, d, $J = 14.6$ Hz), 3.41 (1H, d, $J = 14.6$ Hz), 2.92 (1H, dd, $J = 16.5, 11.5$ Hz), 2.81–2.70 (1H, m), 2.63 (1H, dd, $J = 16.5, 5.2$ Hz), 0.84 (3H, d, $J = 6.4$ Hz); ^{13}C NMR (75 MHz, $\text{DMSO}-d_6$): δ 190.7, 190.6, 174.4, 168.5, 163.5, 156.9, 140.4, 127.5 (2C), 127.4 (2C), 124.9, 103.8, 95.0, 94.3, 90.4, 90.3, 57.0, 56.0, 34.0, 32.5, 27.3, 13.9; HRMS (ESI+) calcd for $[\text{C}_{23}\text{H}_{22}\text{ClO}_6]^+$ 429.1105, found 429.1108.

General procedure for the synthesis of 3'-iodo analogues (**33–35**).

To an ice-cooled solution of griseofulvin (1.4 mmol, 1 equiv.) and *N*-iodosuccinimide (2.1 mmol, 1.5 equiv.) in anhydrous CH_2Cl_2 (15 mL, 0.1 M) under an argon atmosphere, was added triethylsilyl trifluoromethanesulfonate (0.35 mmol 0.25 equiv.). The mixture was stirred at 20 °C for 24 h and then diluted with CH_2Cl_2 (60 mL) and washed with sat. aq. NaHCO_3 (60 mL). The aqueous phase was extracted with CH_2Cl_2 (50 mL), the combined organic phases were dried (MgSO_4) and concentrated. The residue was purified by column chromatography (EtOAc:heptane 1:3) to yield the desired product. When possible the product was re-crystallized from EtOAc/heptane.

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(3'-iodo-2'-methoxy-6'-methyl-cyclohex-2'-ene-4'-one) **33**.

Yield: 126 mg (19%) (white crystals); R_f (EtOAc:heptane 5:1): 0.77; m.p.: 188–190 °C (Litt.¹²); IR (KBr, cm^{-1}): 1717, 1664 (Litt.¹²); ^1H NMR (300 MHz, CDCl_3): δ 5.56 (1H, s), 4.10 (3H, s), 4.01 (3H, s), 3.63 (3H, s), 2.95 (1H, dd, $J = 15.6, 13.3$ Hz), 2.90–2.79 (1H, m), 2.46 (1H, dd, $J = 15.6, 3.6$ Hz), 0.94 (3H, d, $J = 6.4$ Hz); ^{13}C NMR (75 MHz, CDCl_3): δ 196.7, 192.5, 170.8, 170.3, 164.6, 157.0, 106.3, 105.4, 97.1, 91.1, 82.1, 63.1, 61.4, 57.1, 40.1, 36.7, 14.6; HRMS (ESI+) calcd for $[\text{C}_{17}\text{H}_{17}\text{ClIO}_6]^+$ 478.9758, found 478.9761.

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(3'-iodo-6'-methyl-2'-propoxy-cyclohex-2'-ene-4'-one) **34**.

Yield: 49 mg (19%) (yellow crystals); R_f (EtOAc:heptane 5:1): 0.53; m.p.: 98–100 °C (Litt.¹²); IR (KBr, cm^{-1}): 1719, 1618 (Litt.¹²); ^1H NMR (500 MHz, CDCl_3): δ 6.13 (1H, s), 4.14–4.11 (2H, m), 4.03 (3H, s), 4.00 (3H, s), 3.80–3.73 (1H, m), 2.94–2.87 (1H, m), 2.92–2.90 (1H, m), 1.75–1.68 (2H, m), 1.05 (3H, d, $J = 5.8$ Hz), 1.00 (3H, t, $J = 7.4$ Hz); ^{13}C NMR (75 MHz, CDCl_3): δ 196.7, 192.1, 188.0, 172.3, 172.3, 165.5, 158.3, 112.2, 103.8, 97.9, 90.1, 67.9, 57.4, 56.8, 39.4, 38.2, 22.3, 12.2, 10.8; HRMS (ESI+) calcd for $[\text{C}_{19}\text{H}_{21}\text{ClIO}_6]^+$ 507.0071, found 507.0069.

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(3'-iodo-2'-benzyloxy-6'-methyl-cyclohex-2'-ene-4'-one) **35**.

Yield: 56 mg (17%) (white crystals); R_f (EtOAc:heptane 5:1): 0.88; m.p.: 172–175 °C (Litt.¹²); IR (KBr, cm^{-1}): 1715, 1666 (Litt.¹²); ^1H NMR (300 MHz, CDCl_3): δ 7.27–7.22 (3H, m), 7.12–7.10 (2H, m), 5.59 (1H, s), 4.90 (1H, d, $J = 12.3$ Hz), 4.81 (1H, d, $J = 12.3$ Hz), 4.07 (3H, s), 3.95 (3H, s), 2.95 (1H, dd, $J = 15.5, 13.2$ Hz), 2.88–2.81 (1H, m), 2.44 (1H, dd, $J = 15.4, 3.3$ Hz), 0.95 (3H, d, $J = 6.4$ Hz); ^{13}C NMR (75 MHz, CDCl_3): δ 196.7, 192.6, 170.9, 168.9, 164.6, 157.0, 134.6, 128.9 (2C), 128.7, 126.9 (2C), 110.4, 106.5, 106.2, 91.2, 82.0, 71.1, 63.1, 61.4, 40.2, 36.5, 14.6; HRMS (ESI+) calcd for $[\text{C}_{23}\text{H}_{21}\text{ClIO}_6]^+$ 555.0071, found 555.0071; Anal. ($\text{C}_{23}\text{H}_{20}\text{ClIO}_6$) C, H.

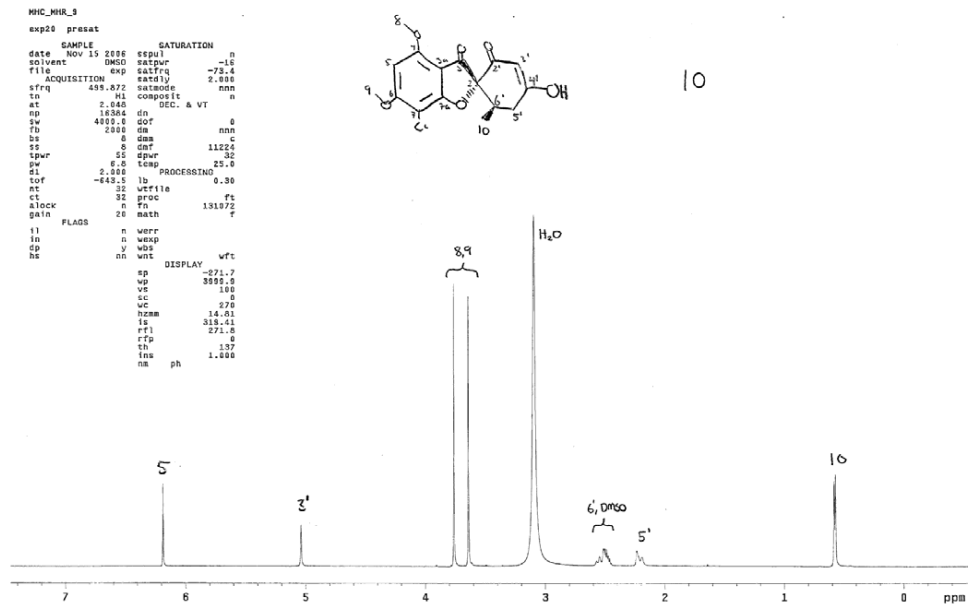
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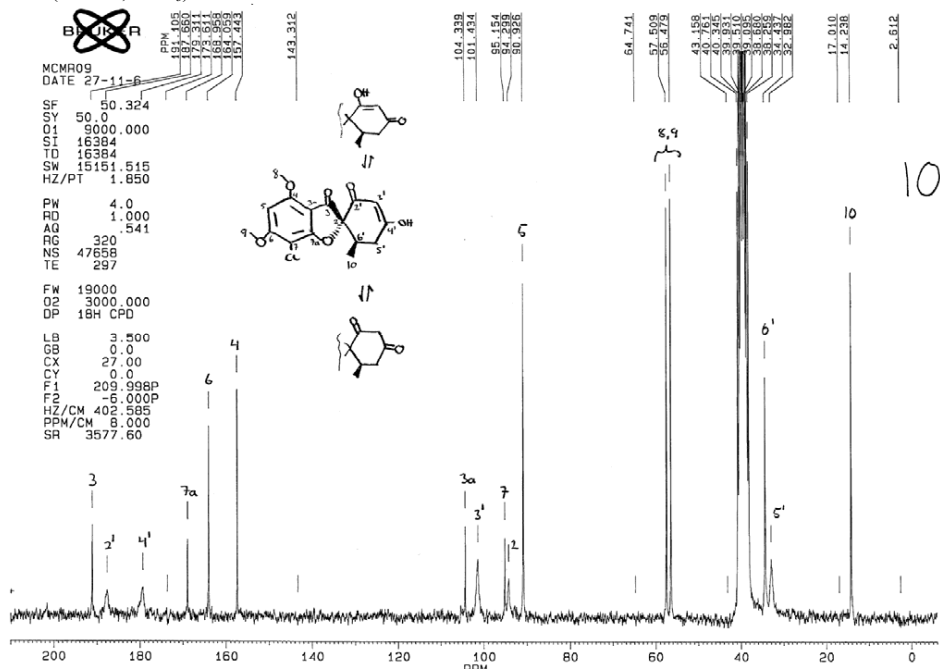
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(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(6'-methyl-cyclohex-2',4'-dione) **2**

¹H NMR (500 MHz, DMSO-*d*₆)

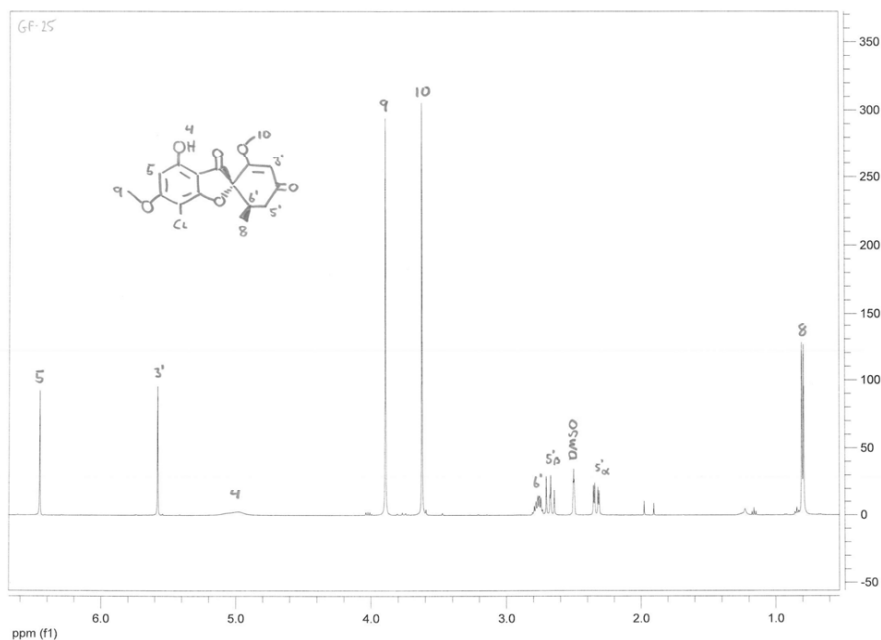


¹³C NMR (50 MHz, CDCl₃)

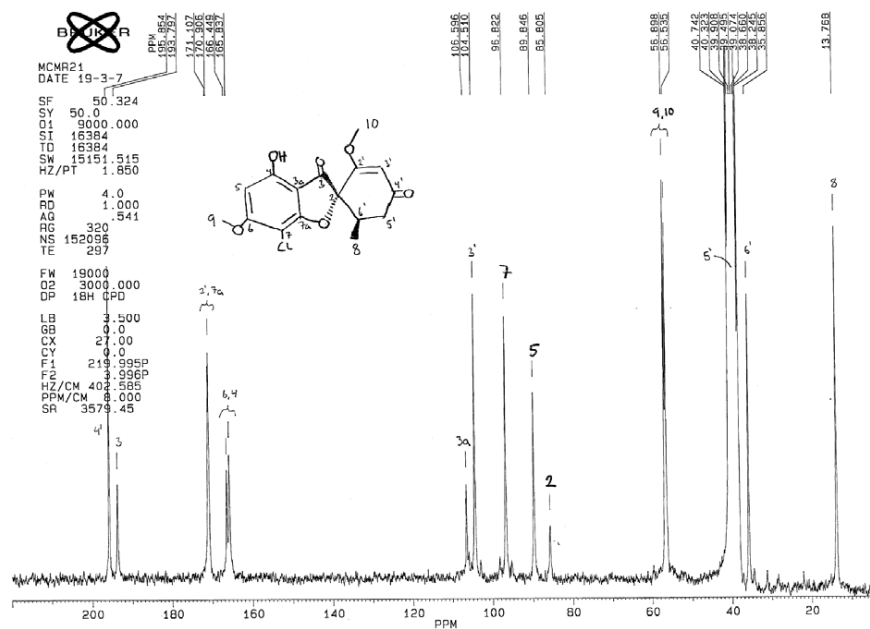


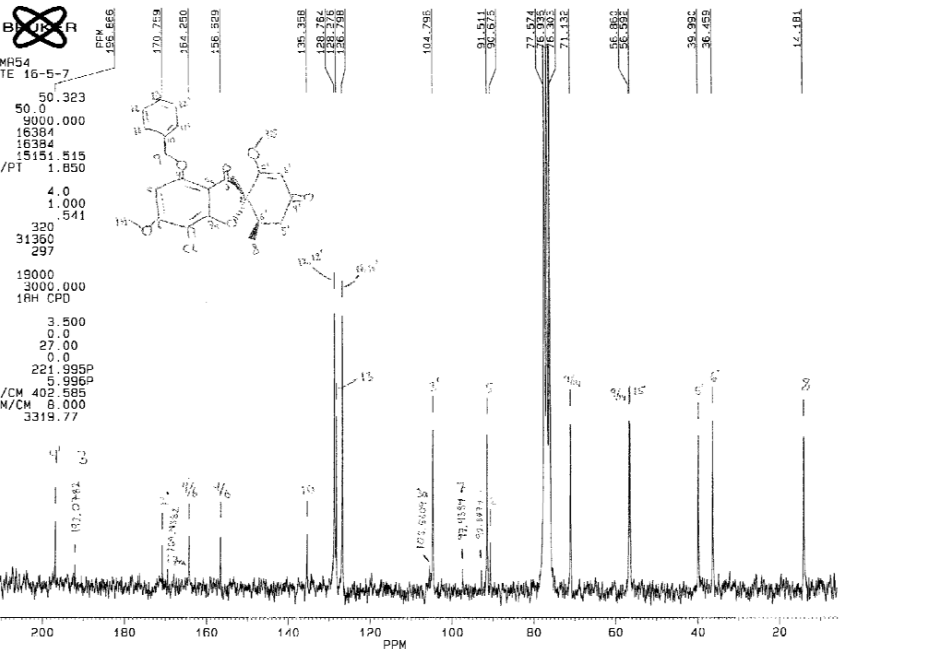
(2*S*,6'*R*)-(7-Chloro-6-methoxy-4-hydroxy-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-en-4'-one) **4**

^1H NMR (500 MHz, $\text{DMSO}-d_6$)



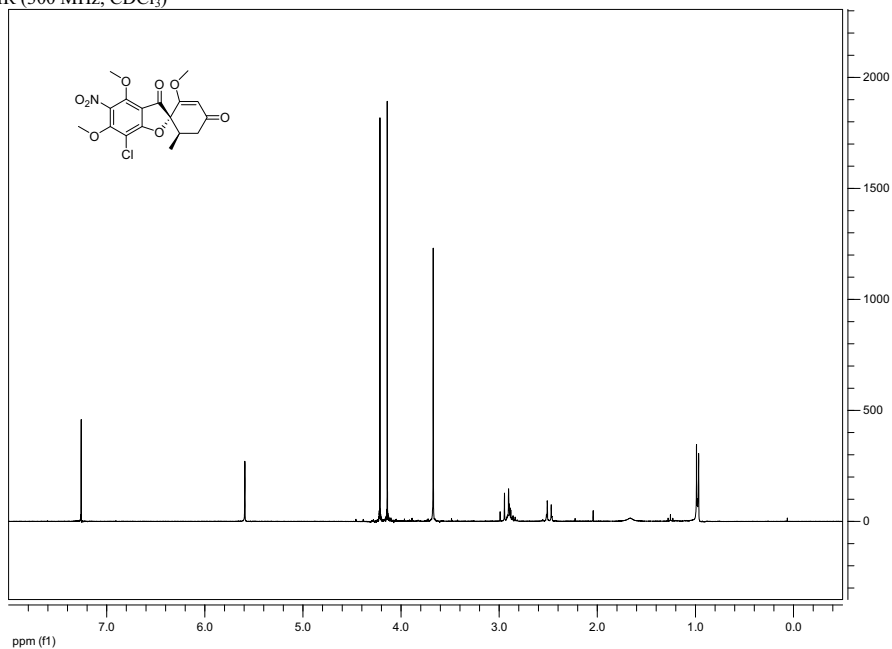
^{13}C NMR (50 MHz, CDCl_3)



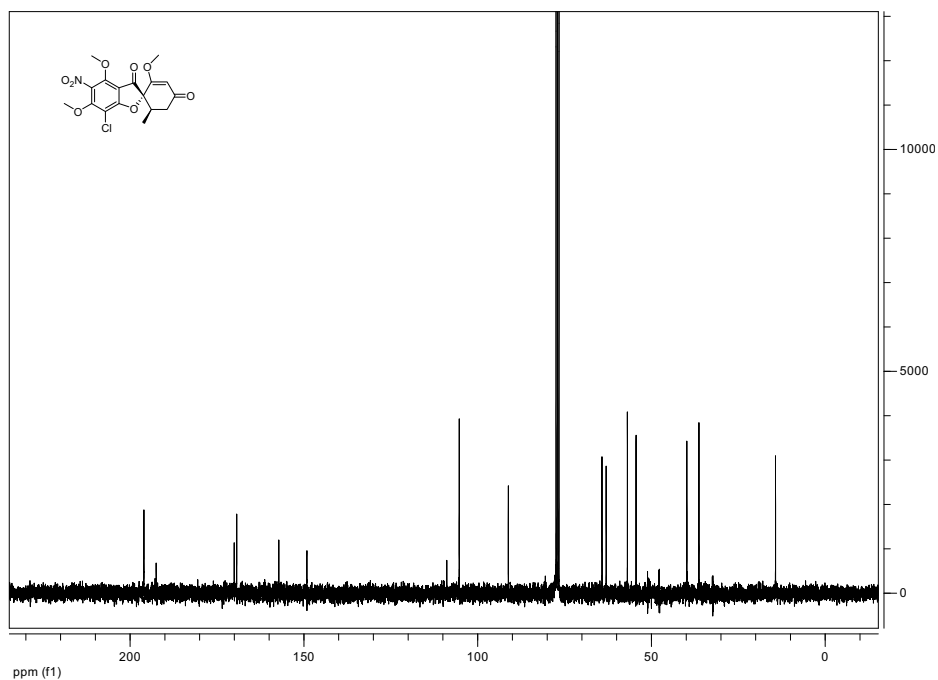


(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-5-nitro-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-ene-4'-one) **7**

^1H NMR (300 MHz, CDCl_3)

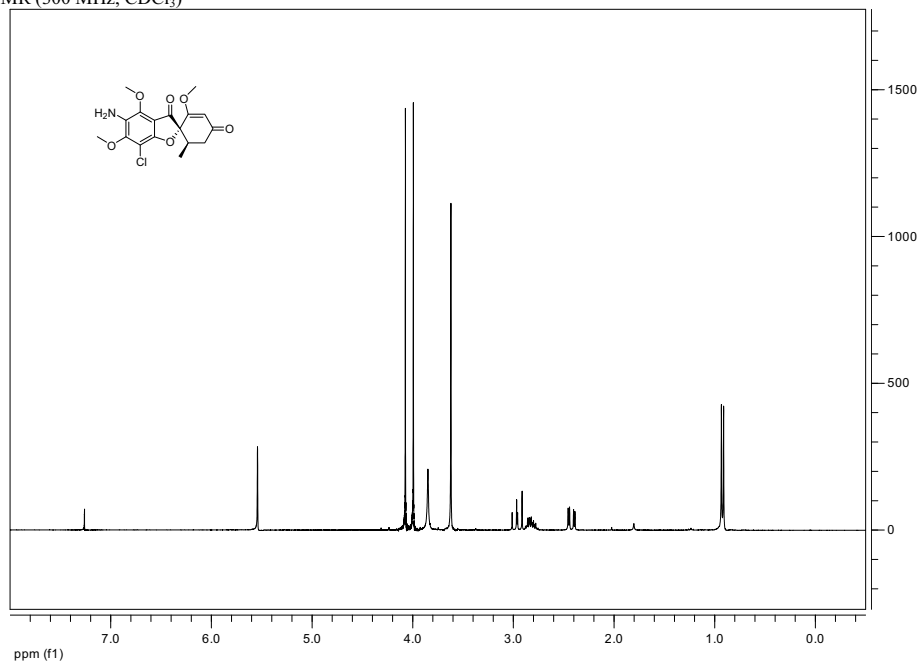


^{13}C NMR (75 MHz, CDCl_3)

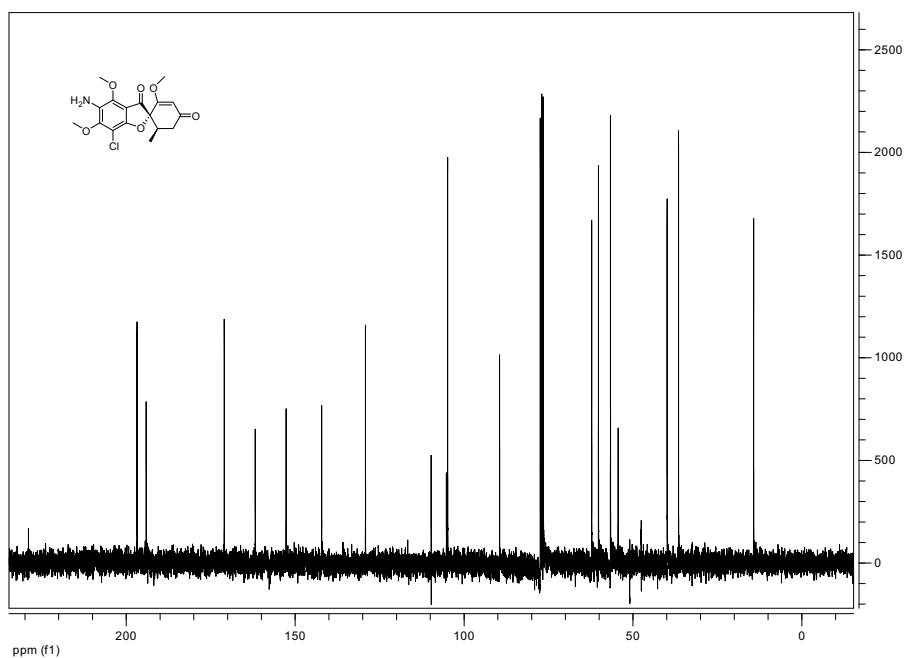


(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-5-amino-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-ene-4'-one) **8**

¹H NMR (300 MHz, CDCl₃)

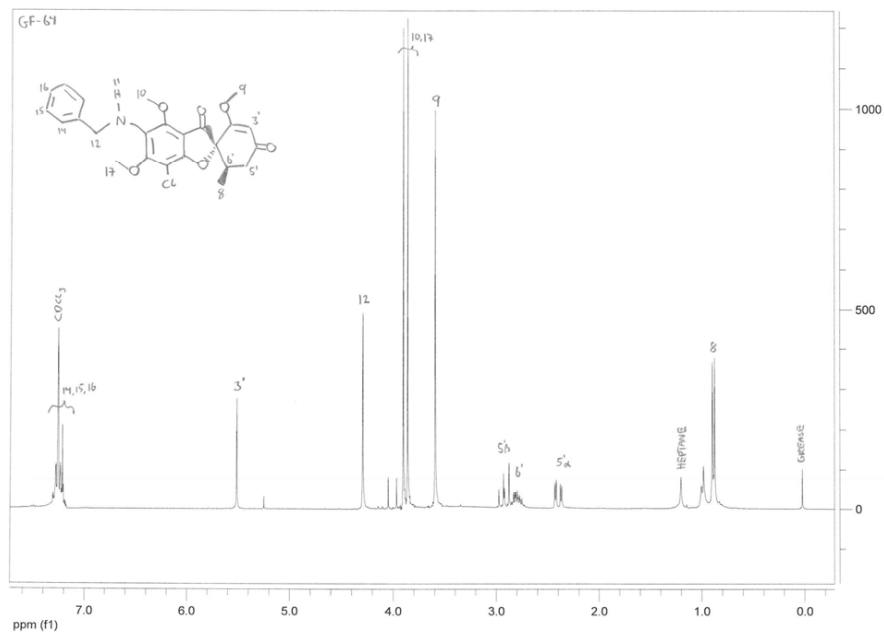


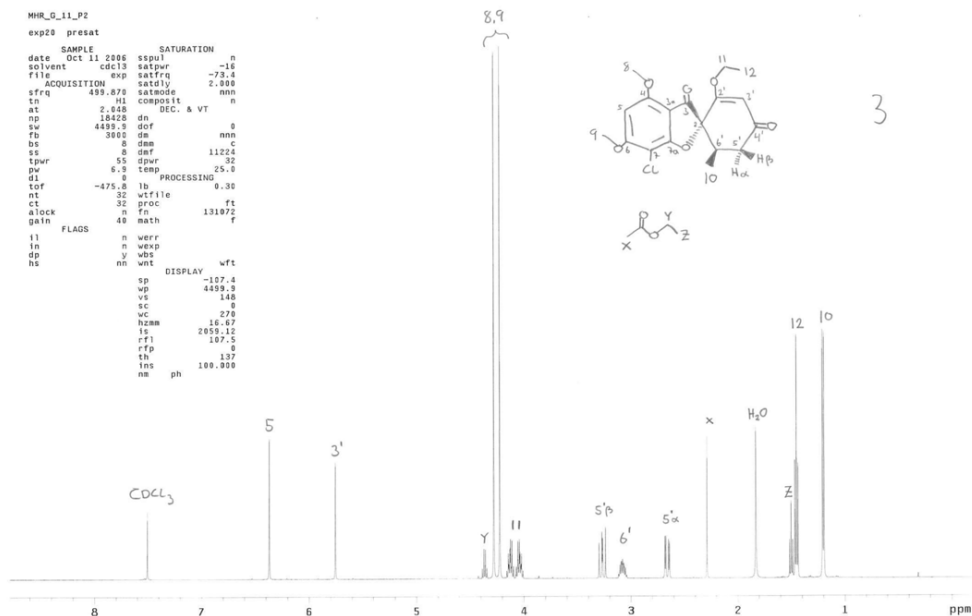
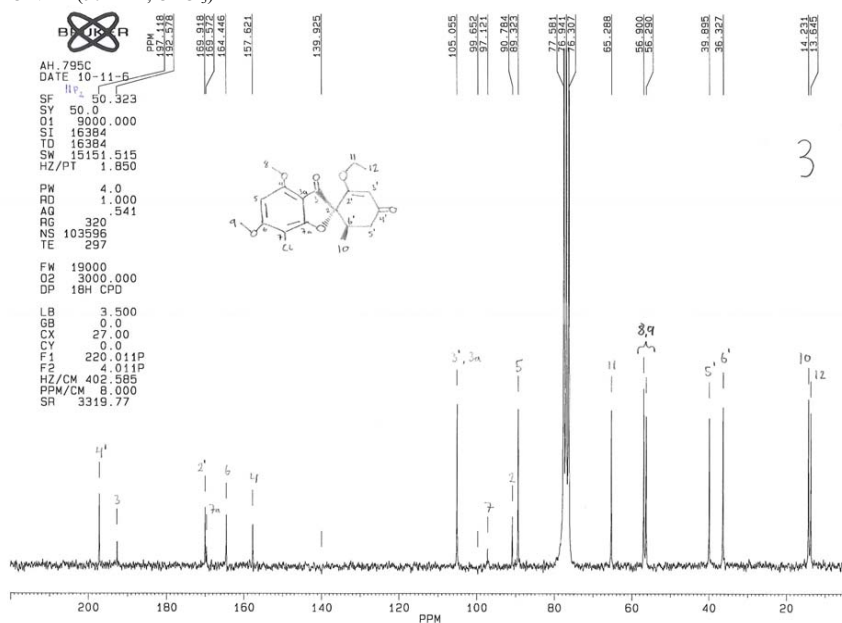
¹³C NMR (75 MHz, CDCl₃)



(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-5-benzylamino-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-ene-4'-one) **9**

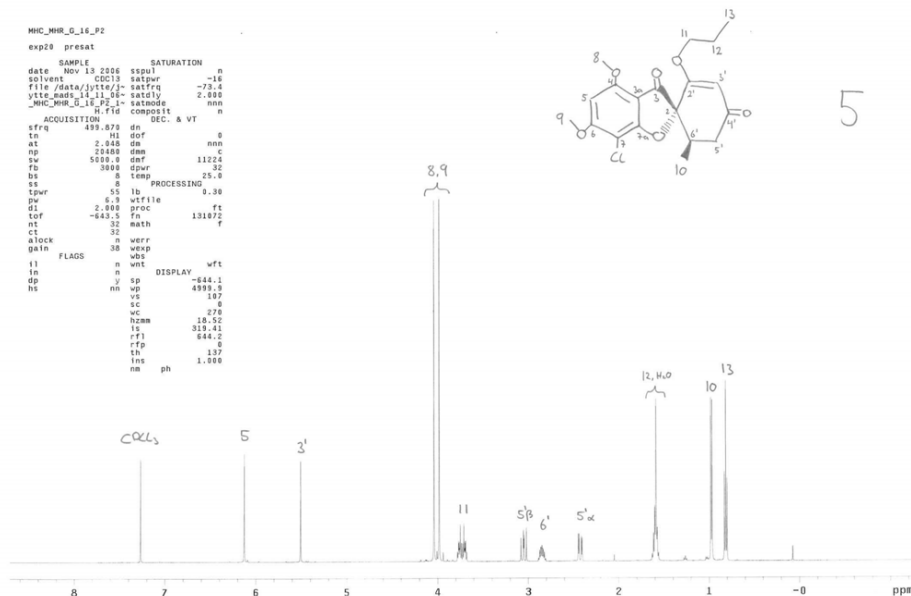
^1H NMR (300 MHz, CDCl_3)



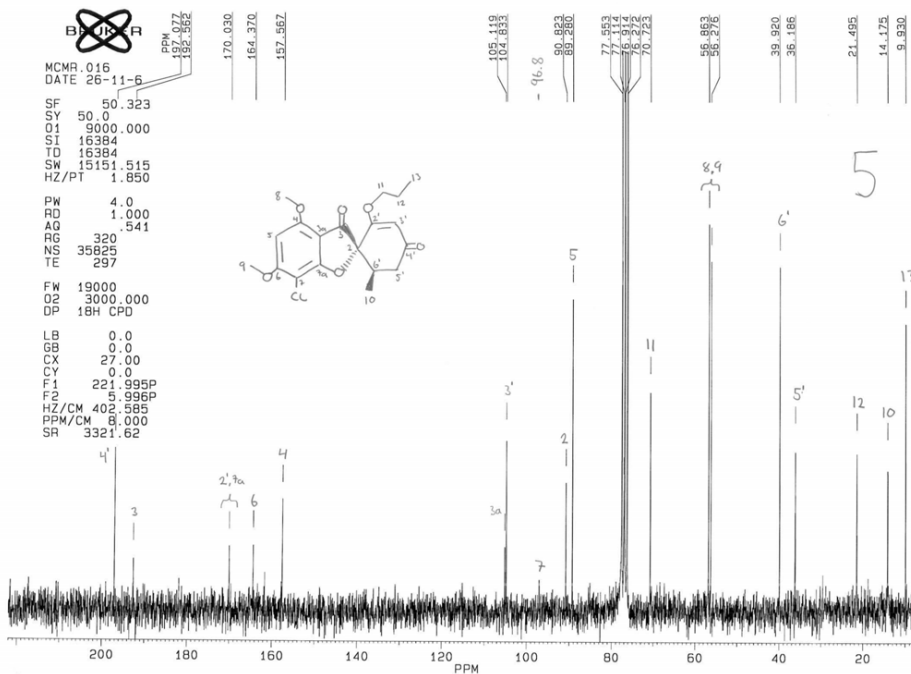
(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(2'-ethoxy-6'-methyl-cyclohex-2'-en-4'-on) **10**¹H NMR (500 MHz, CDCl₃)¹³C NMR (50 MHz, CDCl₃)

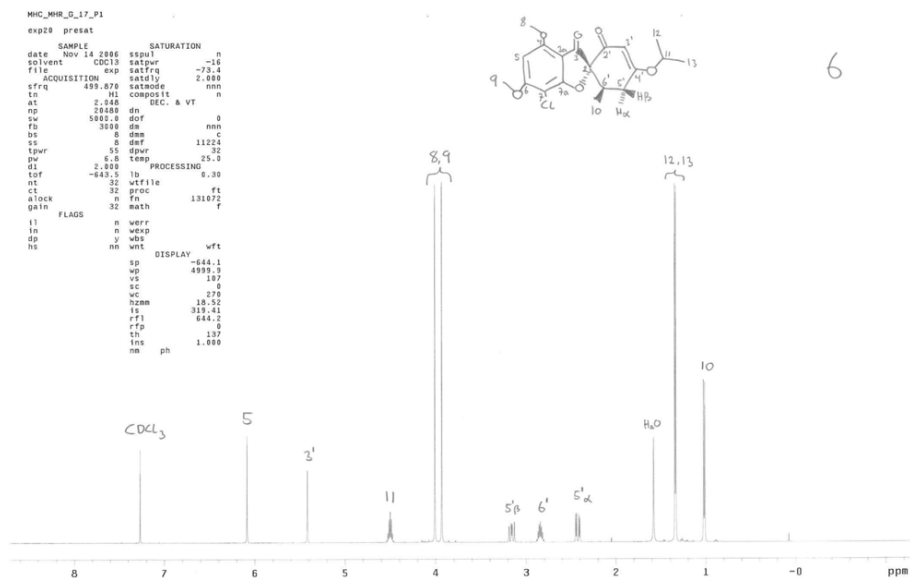
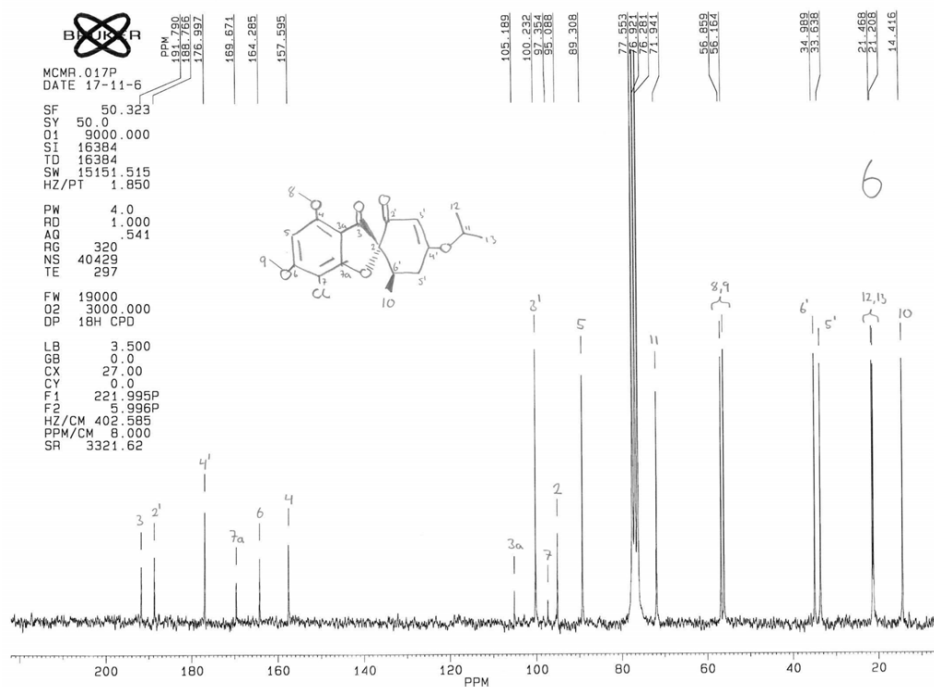
(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(6'-methyl-2'-propoxy-cyclohex-2'-en-4'-on) **11**

¹H NMR (500 MHz, CDCl₃)



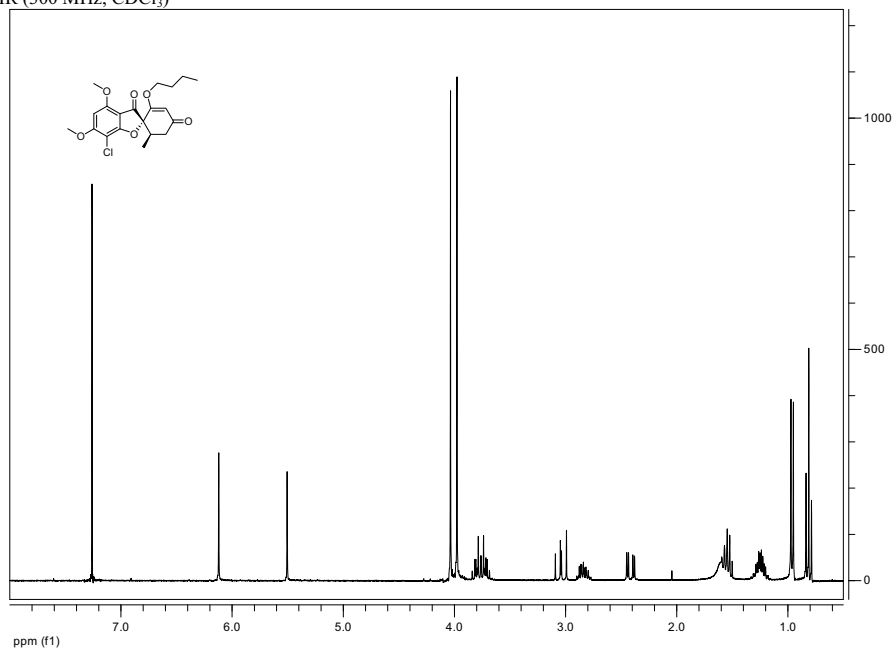
¹³C NMR (50 MHz, CDCl₃)



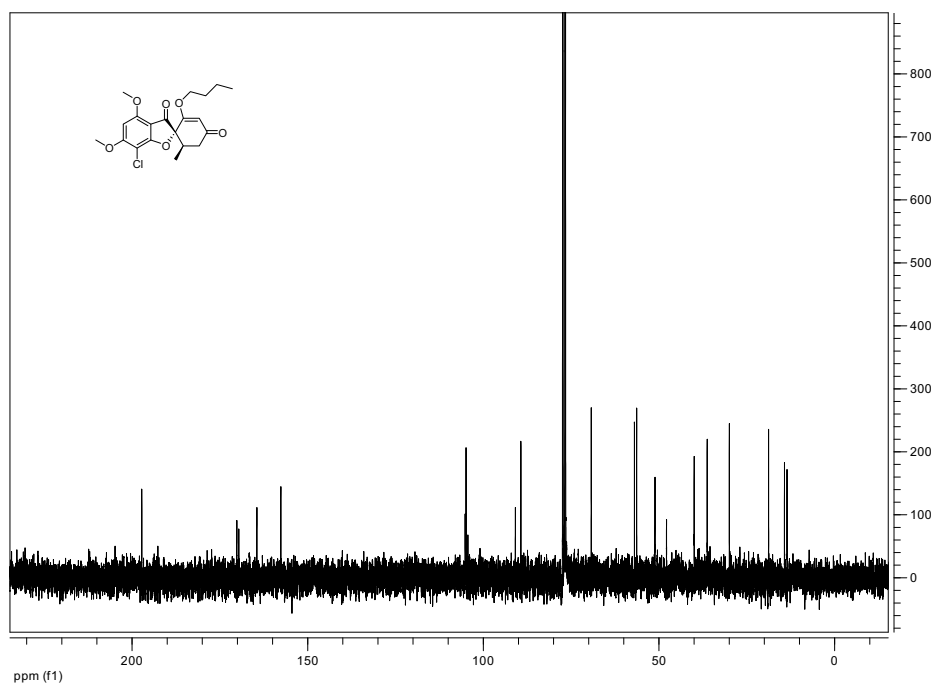
(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(2'-isopropoxy-6'-methyl-cyclohex-2'-en-4'-on) **12**¹H NMR (500 MHz, CDCl₃)¹³C NMR (50 MHz, CDCl₃)

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-butoxy-6'-methyl-cyclohex-2'-ene-4'-one) **13**

^1H NMR (300 MHz, CDCl_3)

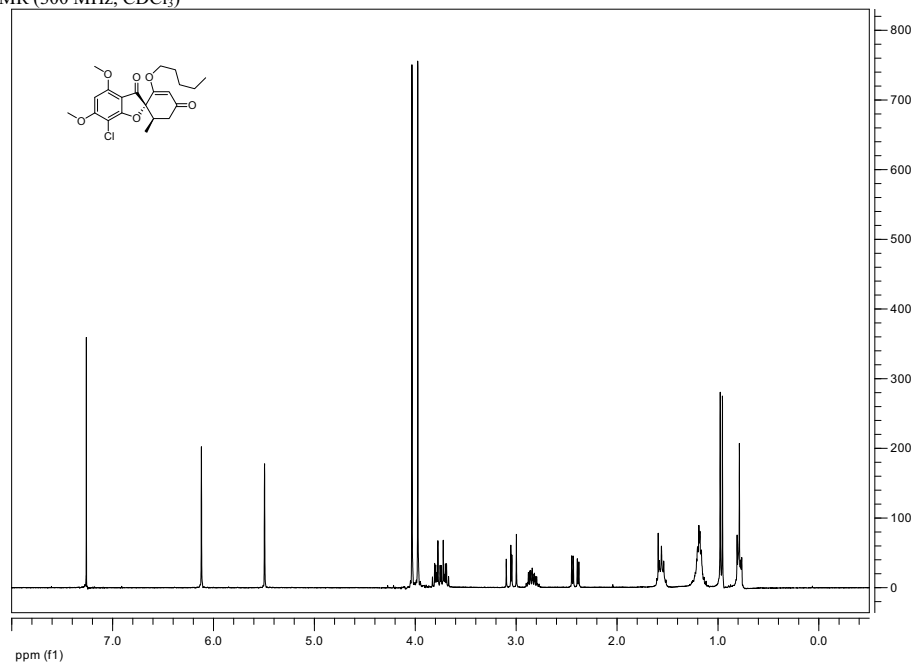


^{13}C NMR (75 MHz, CDCl_3)

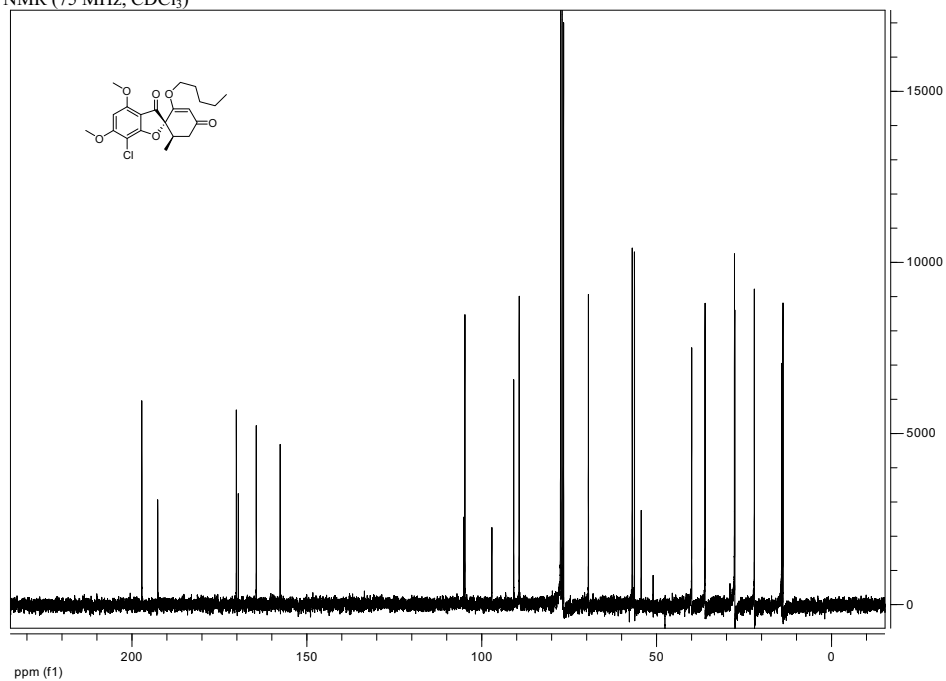


(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(6'-methyl-2'-pentoxy-cyclohex-2'-ene-4'-one) **14**

^1H NMR (300 MHz, CDCl_3)

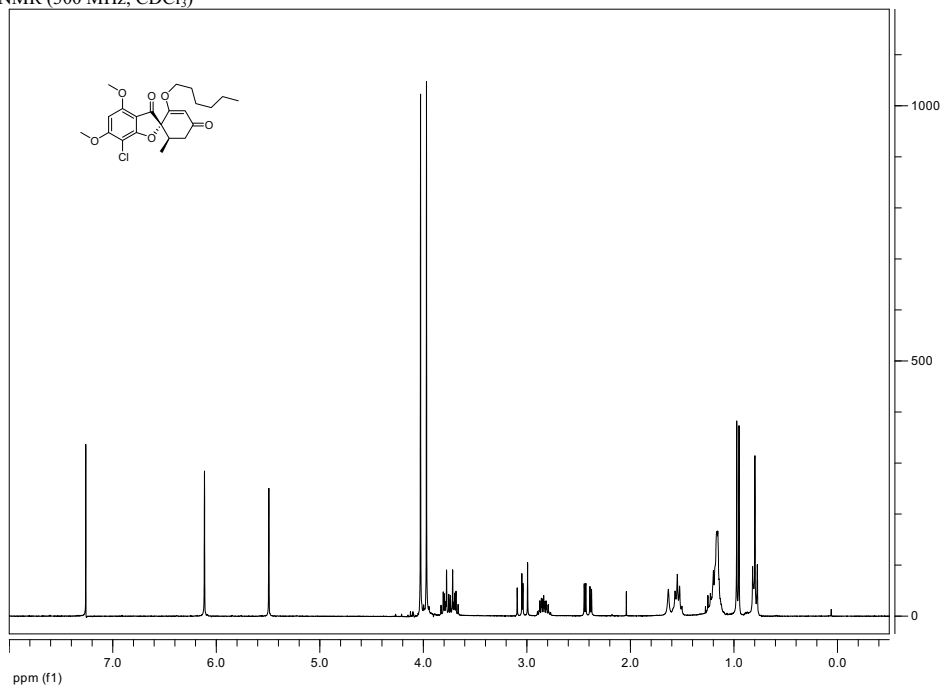


^{13}C NMR (75 MHz, CDCl_3)

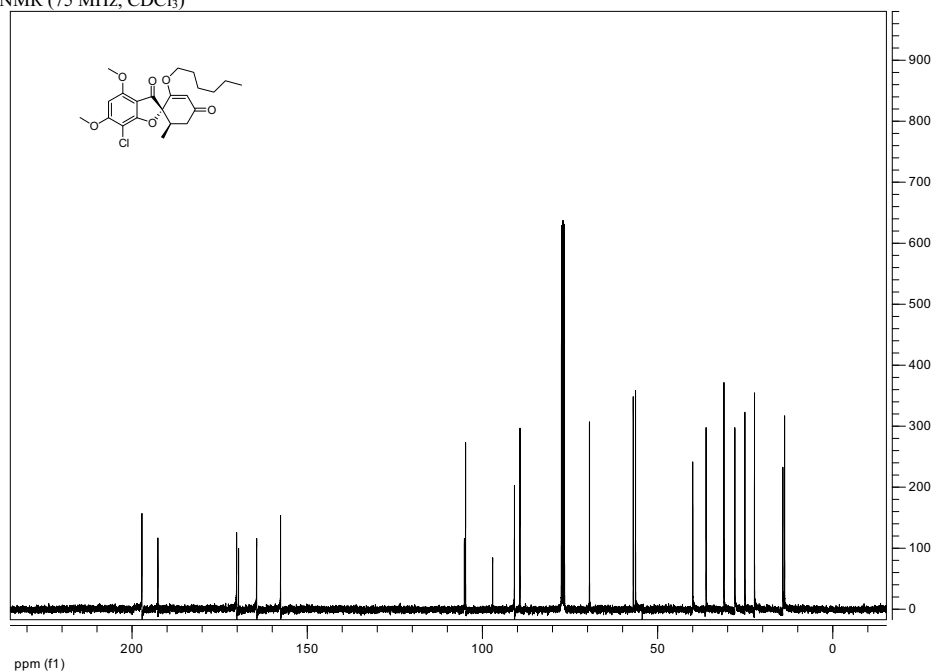


(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-hexoxy-6'-methyl-cyclohex-2'-ene-4'-one) **15**

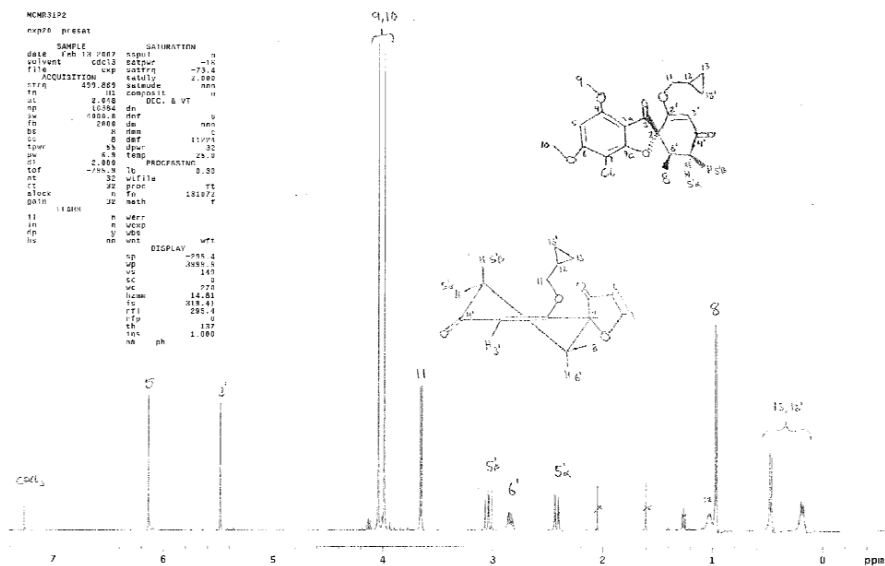
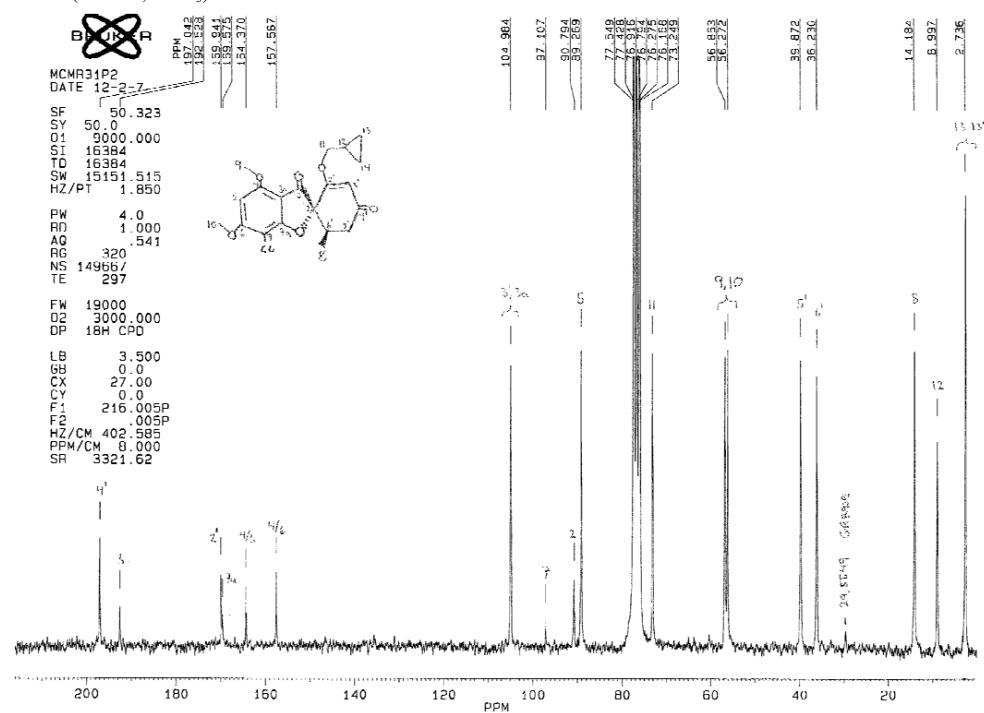
¹H NMR (300 MHz, CDCl₃)

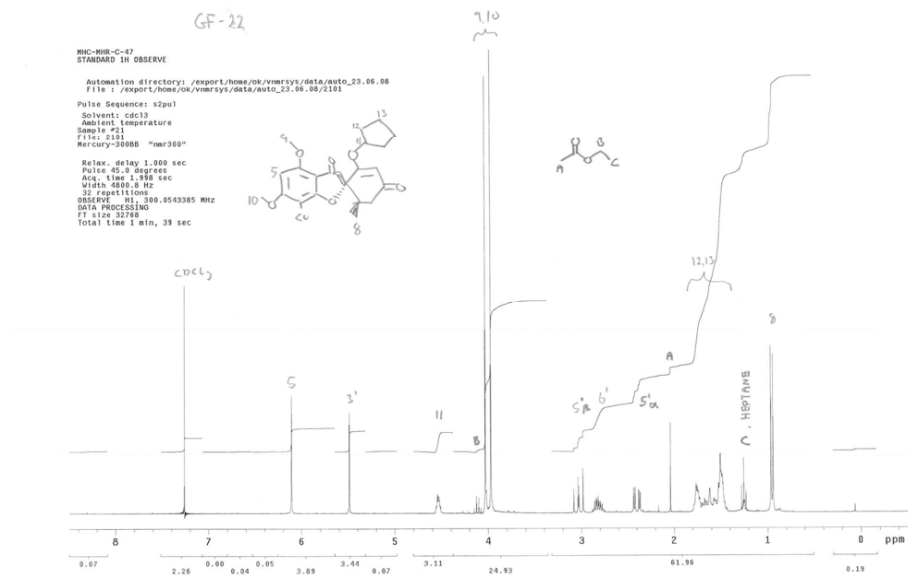
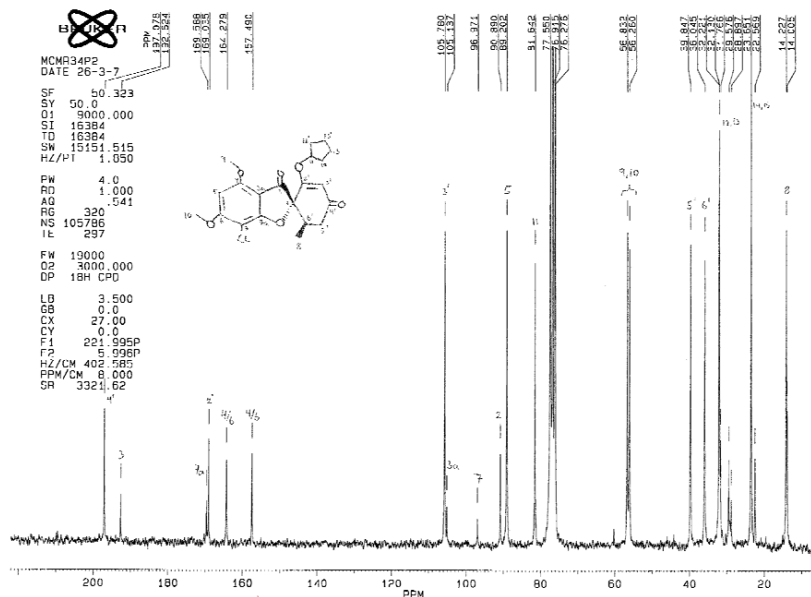


¹³C NMR (75 MHz, CDCl₃)



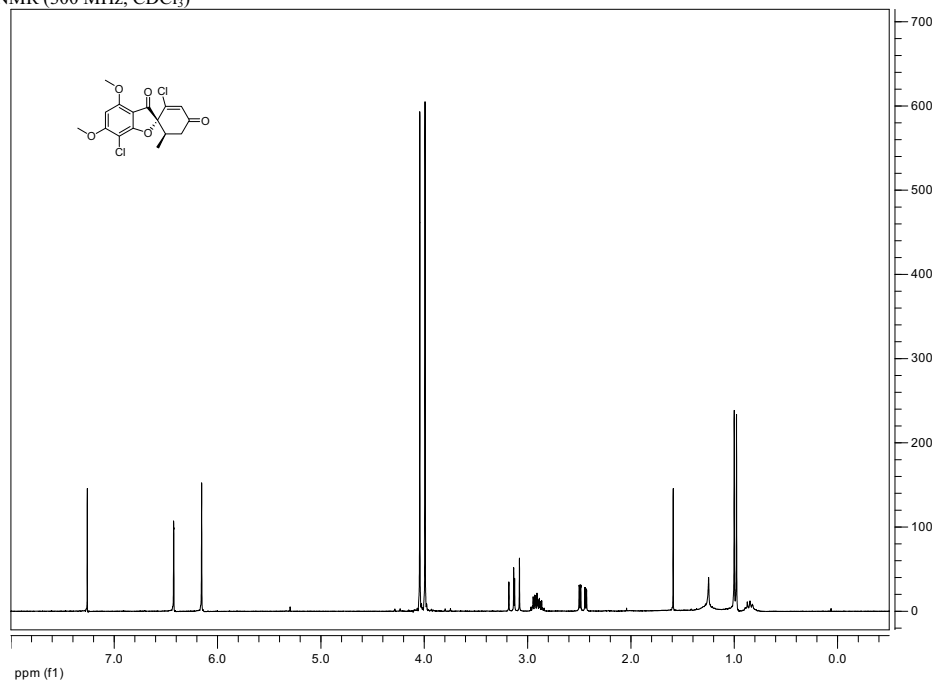
(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(2'-cyclopropylmethoxy-6'-methyl-cyclohex-2'-en-4'-one) **16**

¹H NMR (500 MHz, CDCl₃) ^{13}C NMR (50 MHz, CDCl_3)

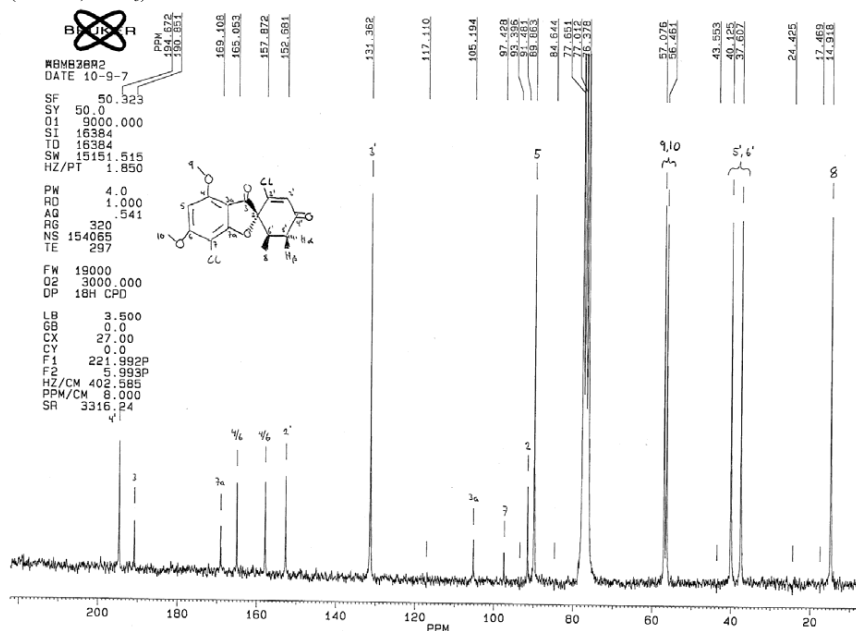
¹H NMR (500 MHz, CDCl₃) ^{13}C NMR (50 MHz, CDCl_3)

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-chloro-6'-methyl-cyclohex-2'-ene-4'-one) **18**

¹H NMR (300 MHz, CDCl₃)

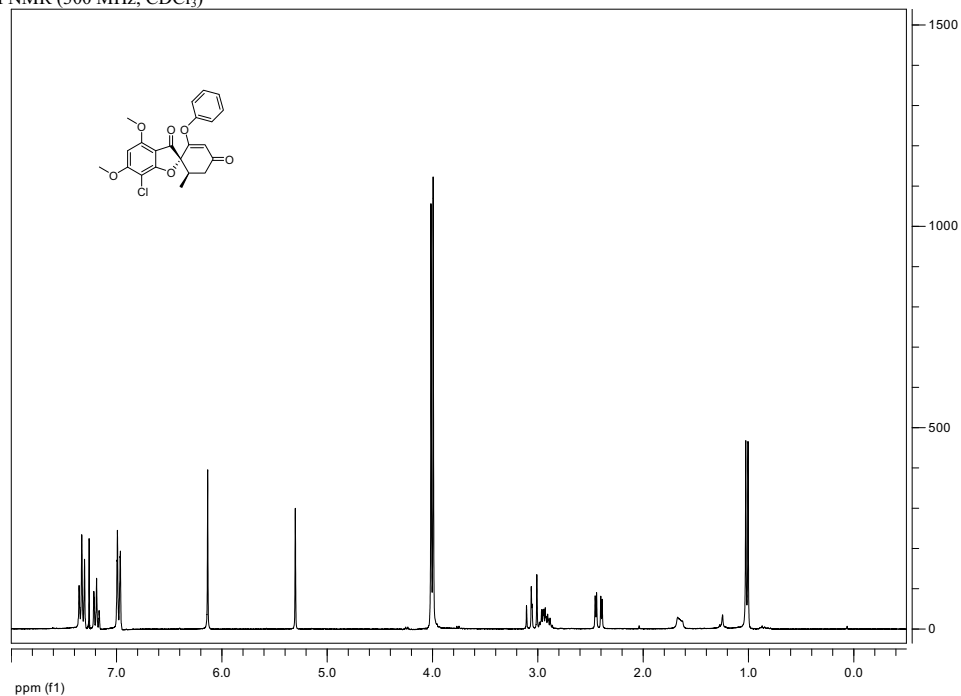


¹³C NMR (50 MHz, CDCl₃)

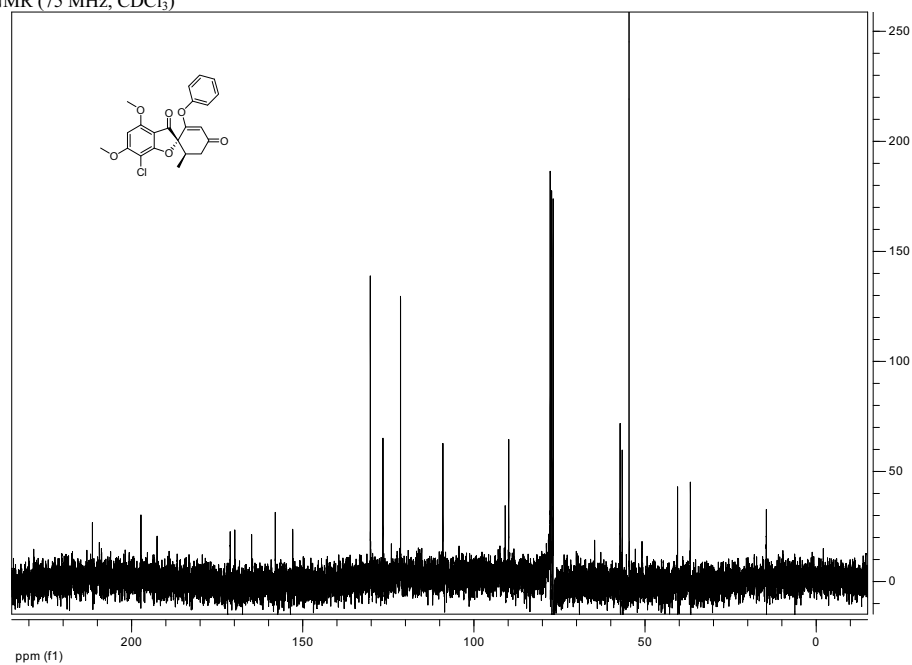


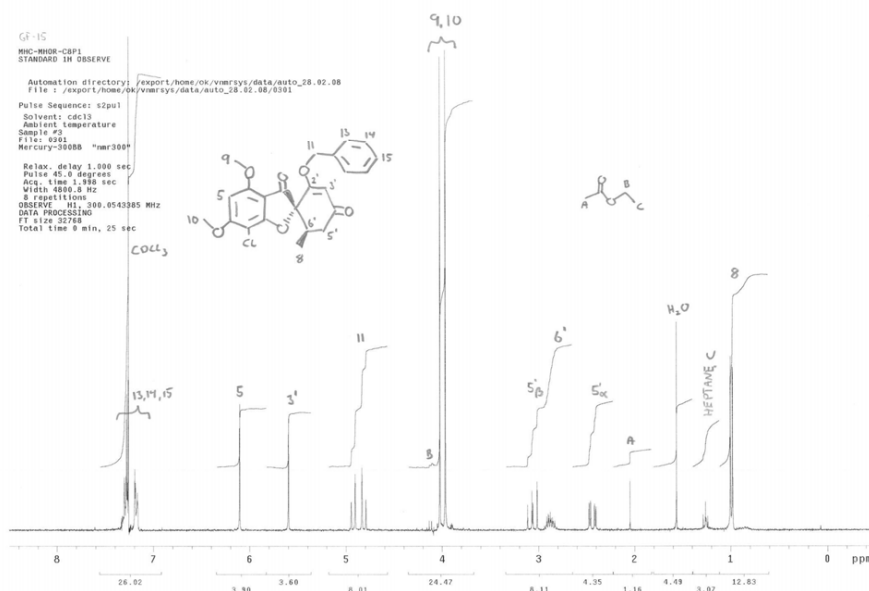
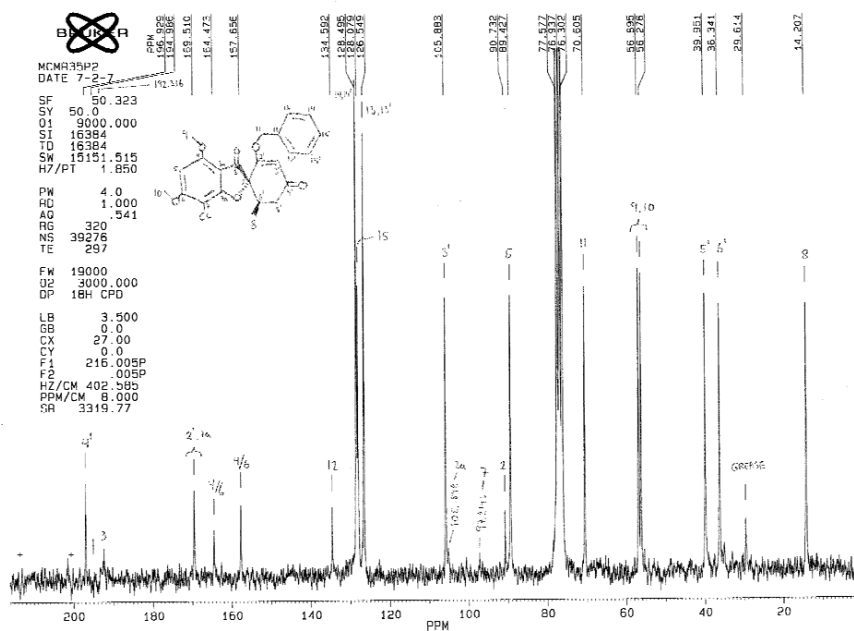
(2S,6'R)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(6'-methyl-2'-phenoxy-cyclohex-2'-ene-4'-one) **19**

^1H NMR (300 MHz, CDCl_3)



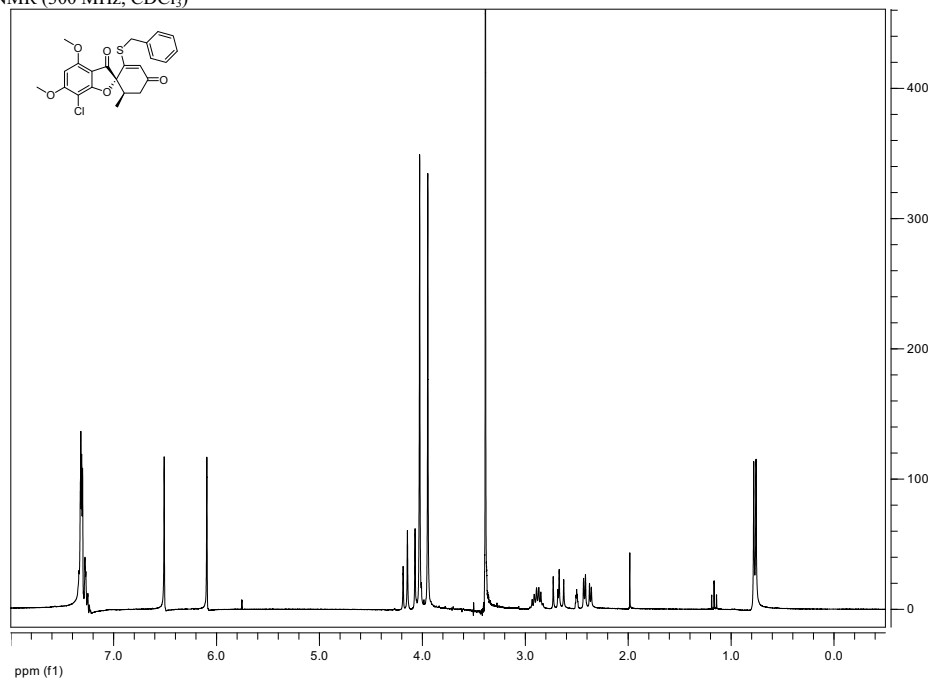
^{13}C NMR (75 MHz, CDCl_3)



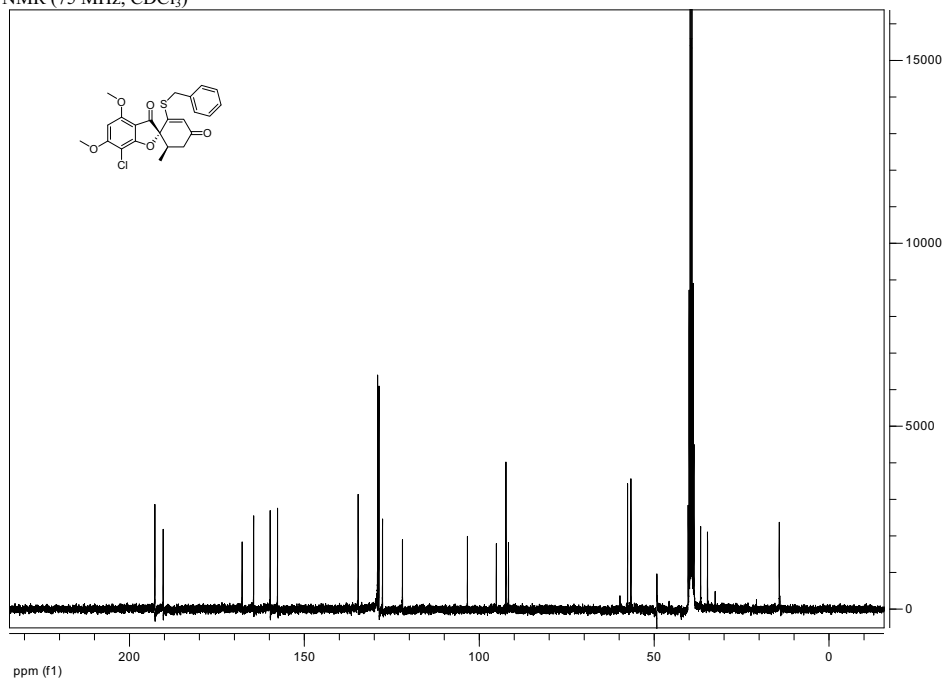
(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(2'-benzyloxy-6'-methyl-cyclohex-2'-en-4'-one) **20**¹H NMR (300 MHz, CDCl₃)¹³C NMR (75 MHz, CDCl₃)

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-benzylthio-6'-methyl-cyclohex-2'-ene-4'-one) **21**

^1H NMR (300 MHz, CDCl_3)

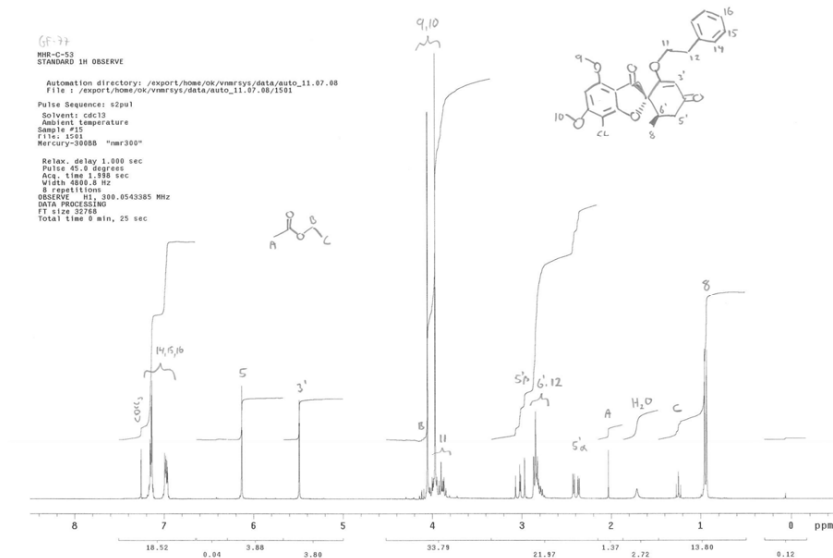


^{13}C NMR (75 MHz, CDCl_3)

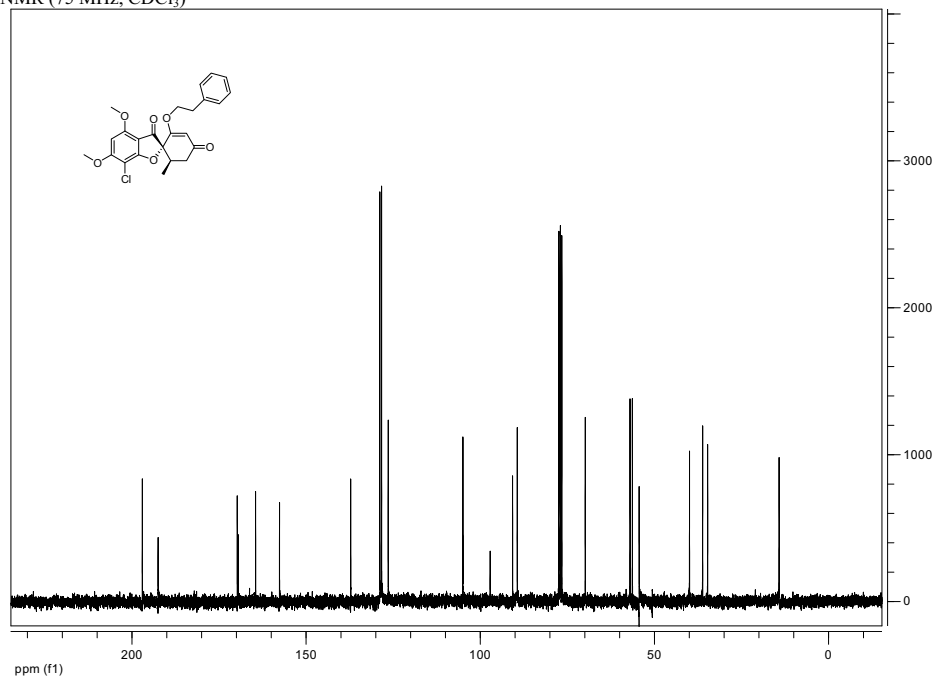


(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(2'-(2-phenylethoxy)-6'-methyl-cyclohex-2'-en-4'-one) **22**

^1H NMR (300 MHz, CDCl_3)

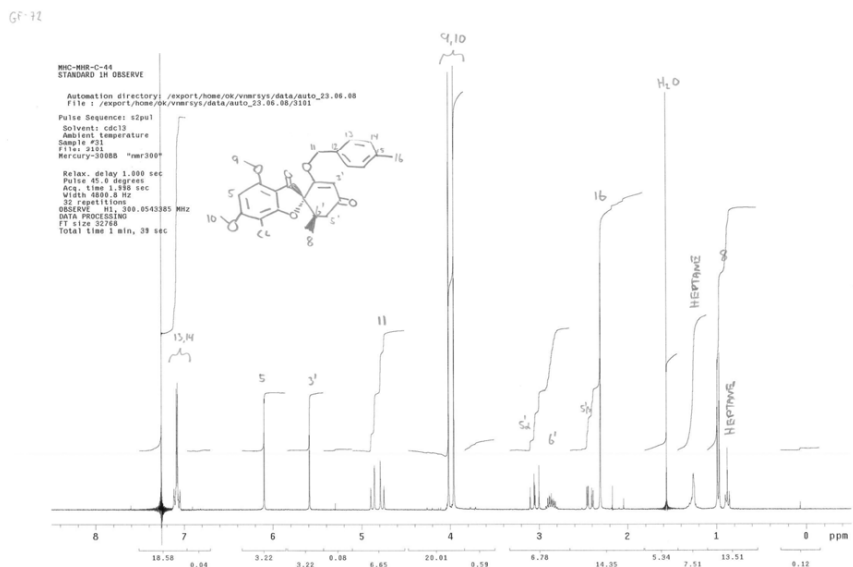


^{13}C NMR (75 MHz, CDCl_3)



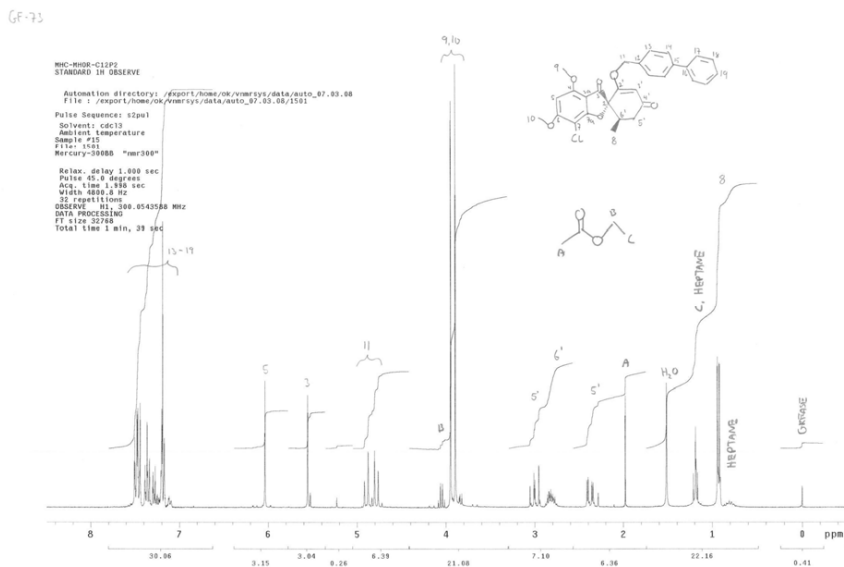
(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-4-methylbenzyloxy-6'-methyl-cyclohex-2'-ene-4'-one) **23**

^1H NMR (300 MHz, CDCl_3)

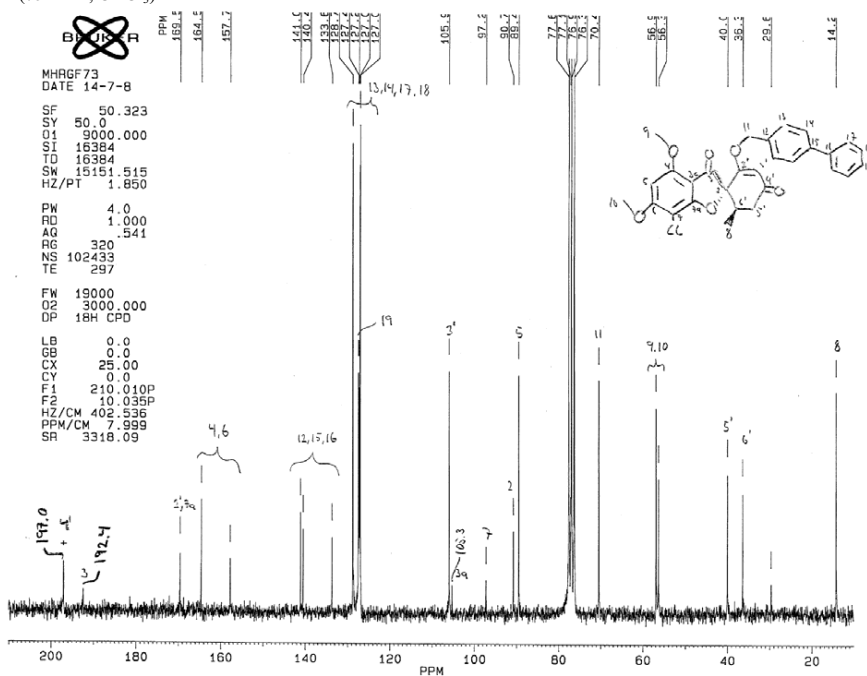


(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-(4-biphenylmethoxy)-6'-methyl-cyclohex-2'-ene-4'-one) **24**

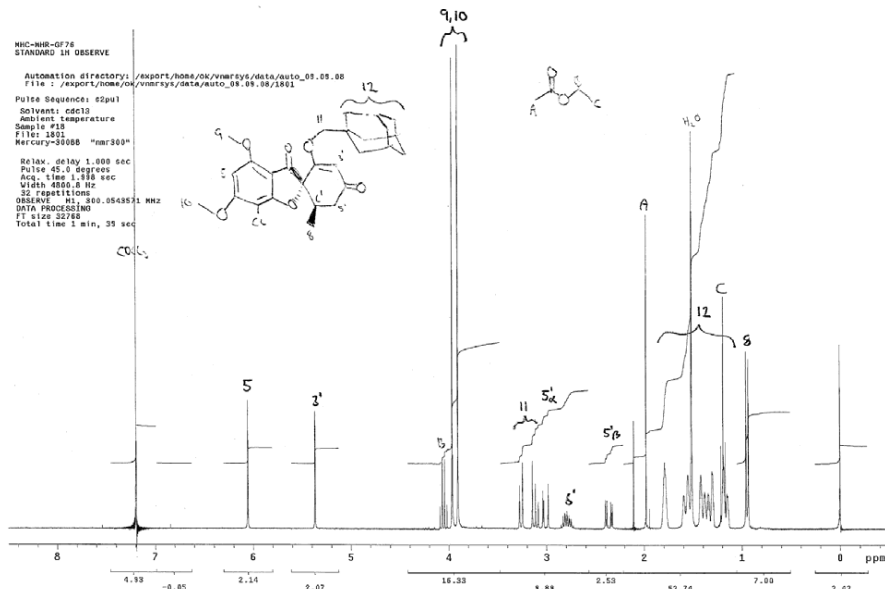
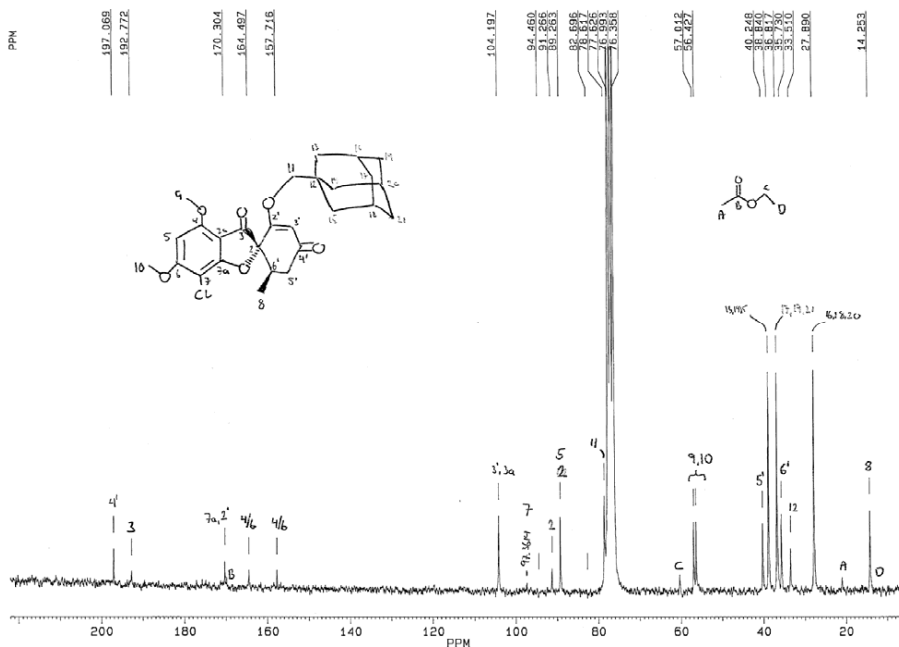
¹H NMR (300 MHz, CDCl₃)



¹³C NMR (75 MHz, CDCl₃)

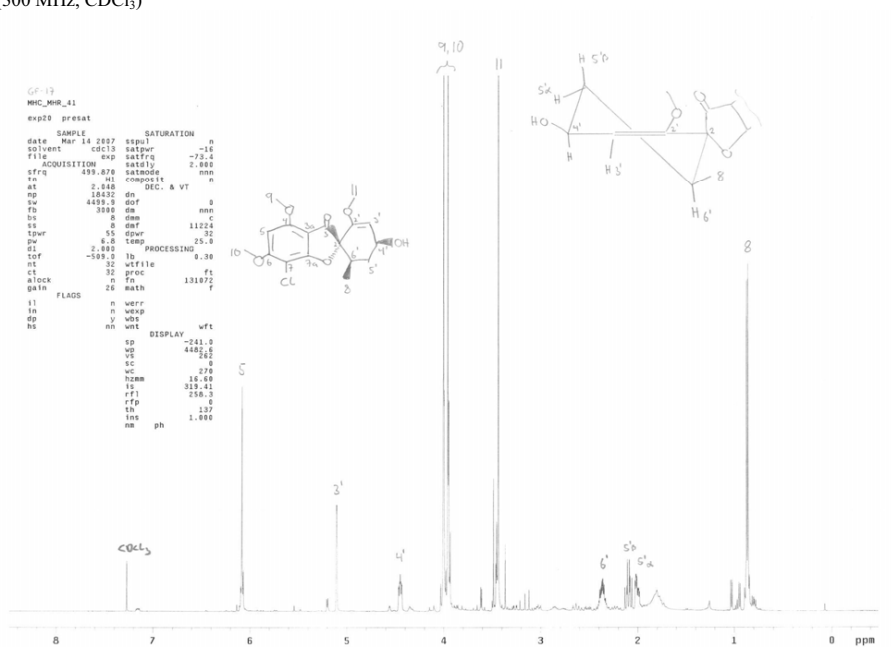


(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-(1-adamantylmethoxy)-6'-methyl-cyclohex-2'-ene-4'-one)

¹H NMR (300 MHz, CDCl₃) ^{13}C NMR (50 MHz, CDCl_3)

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-en-4'-ol) **26**

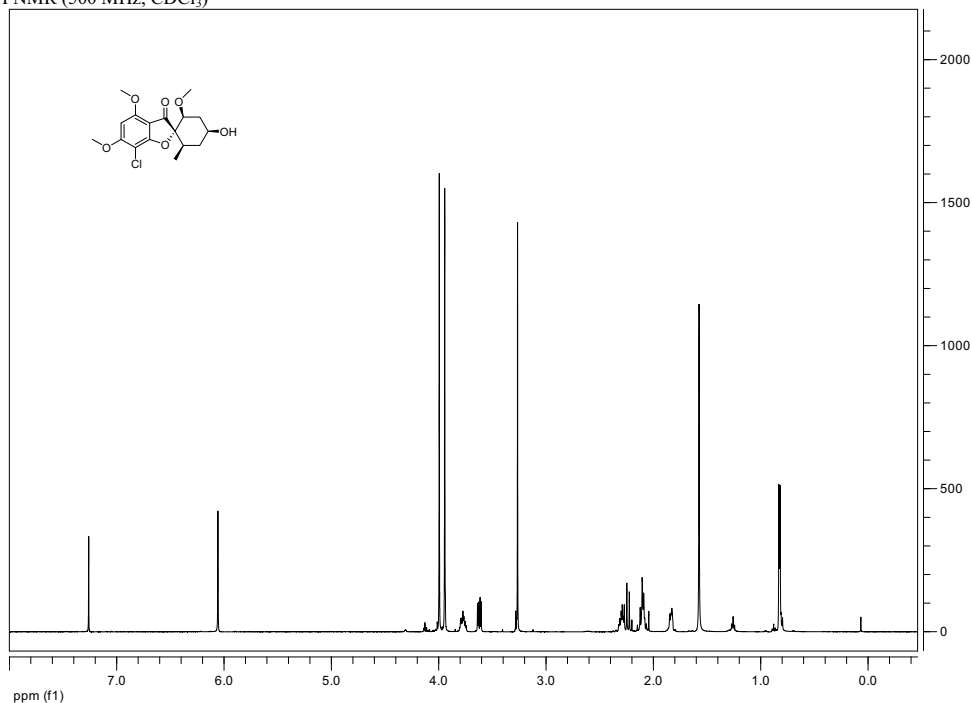
¹H NMR (300 MHz, CDCl₃)



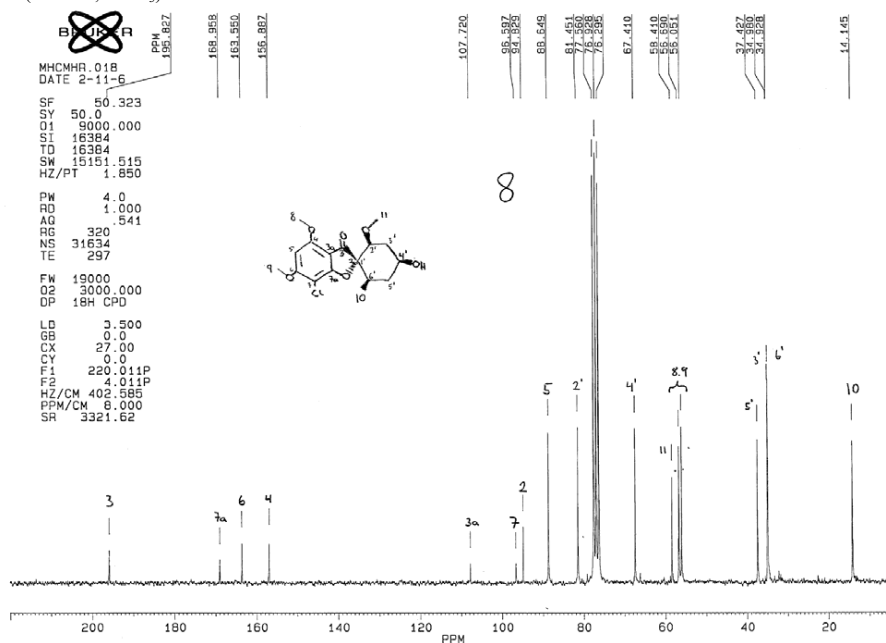
Carbon shifts from gHSQC, gHMBC

(2*S*,2'*S*,4'*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohexan-4'-ol) **27**

¹H NMR (500 MHz, CDCl₃)

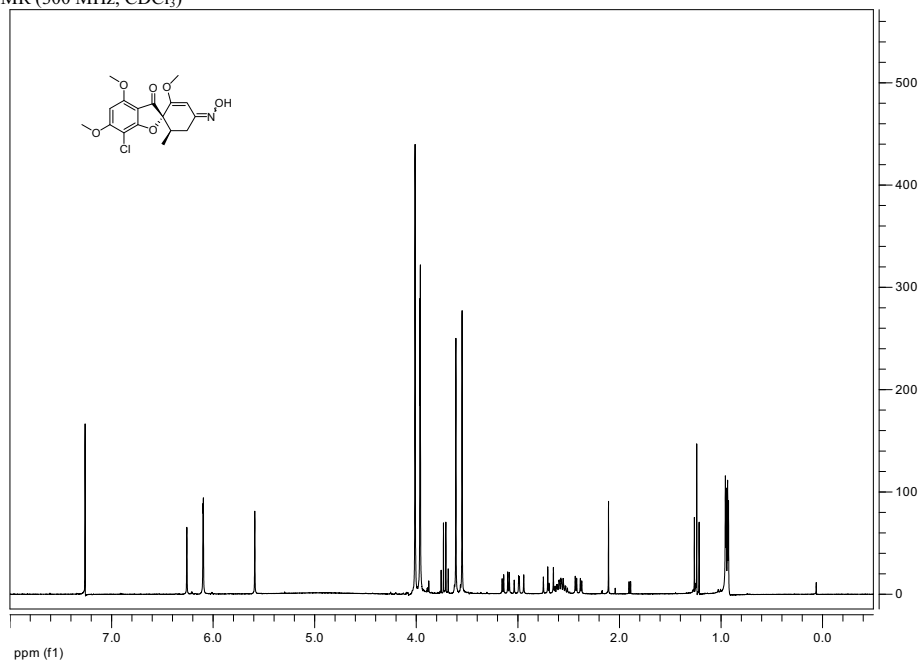


¹³C NMR (50 MHz, CDCl₃)

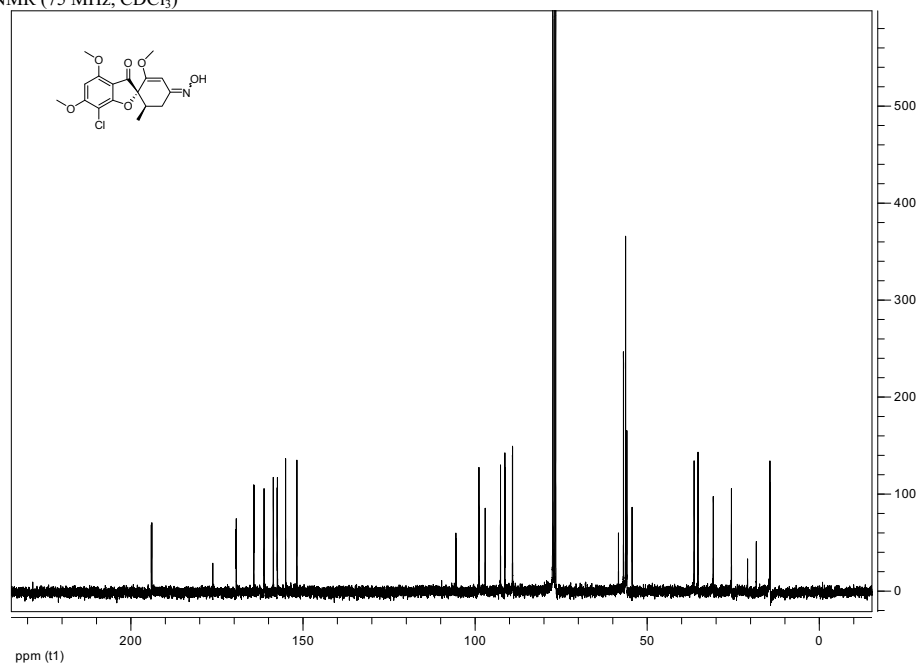


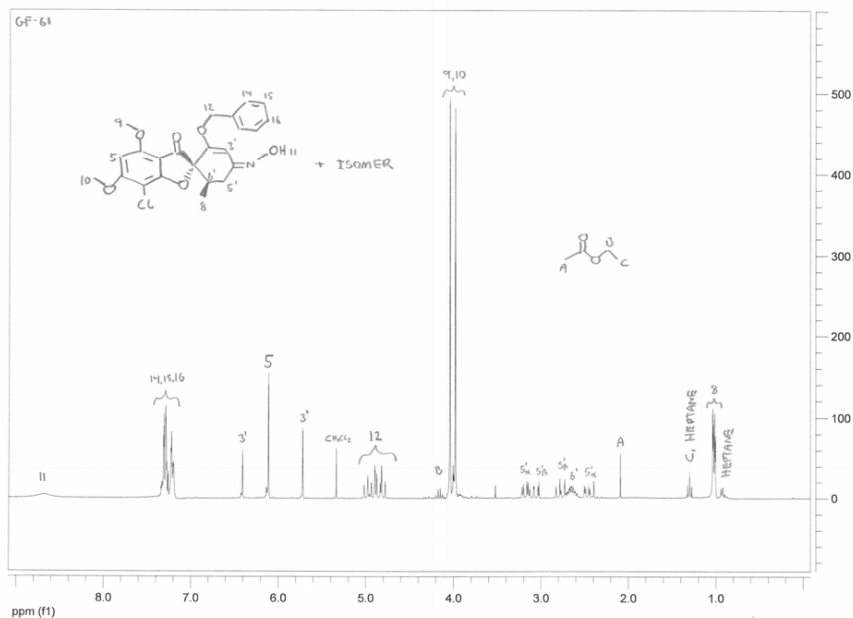
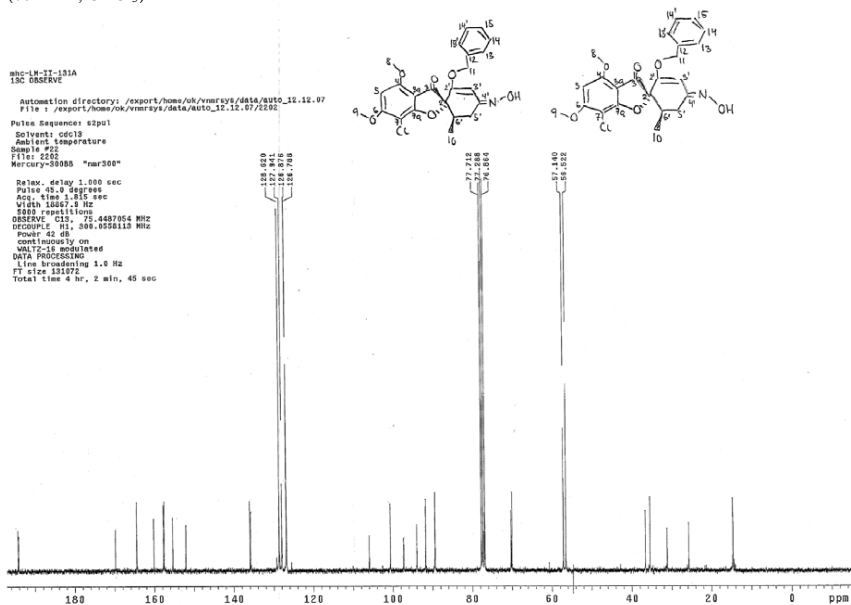
(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-ene-4'-one-4'-oxime) **28**

¹H NMR (300 MHz, CDCl₃)

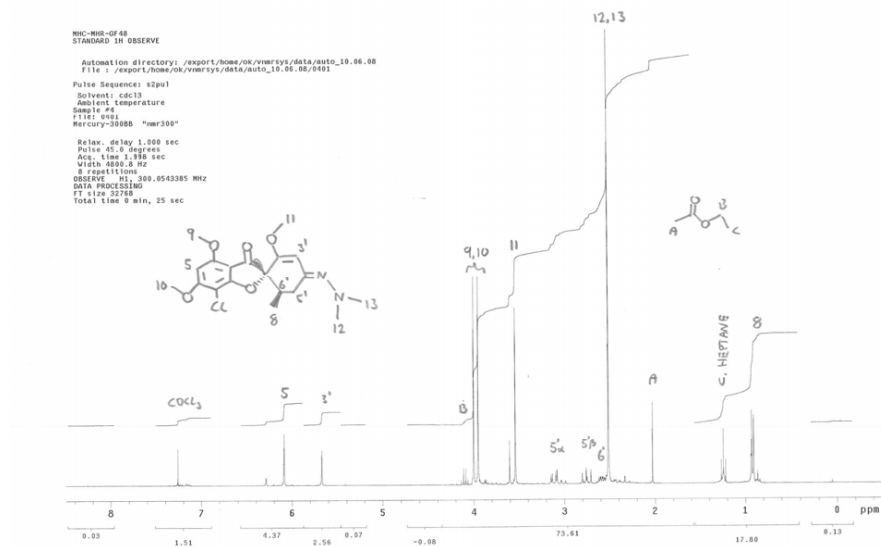
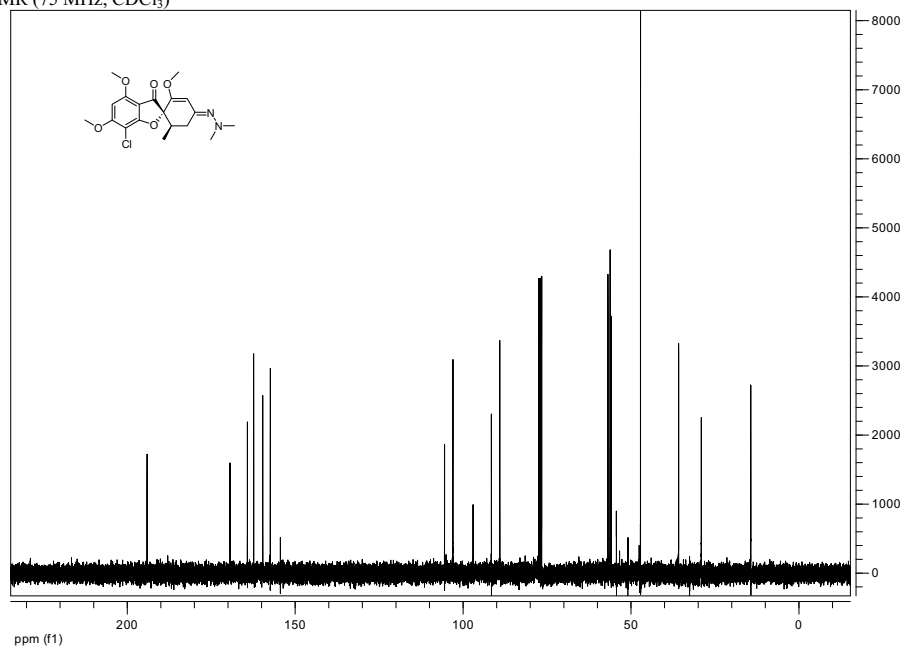


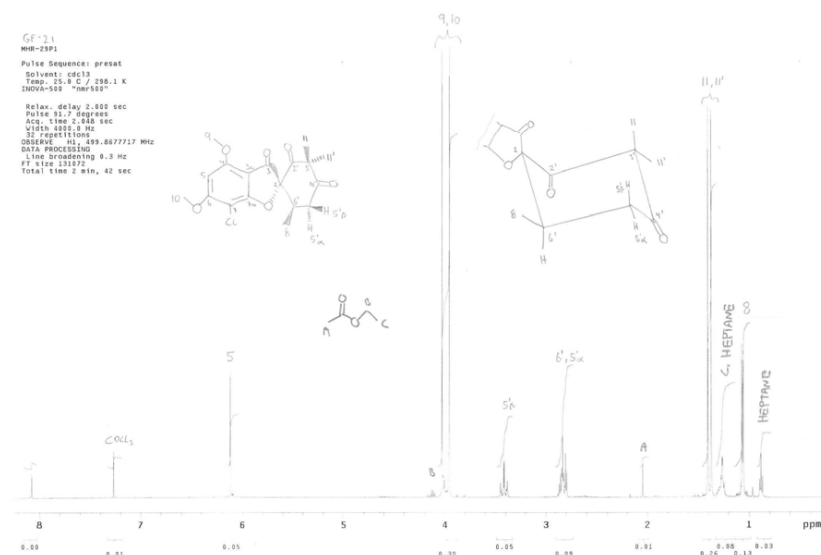
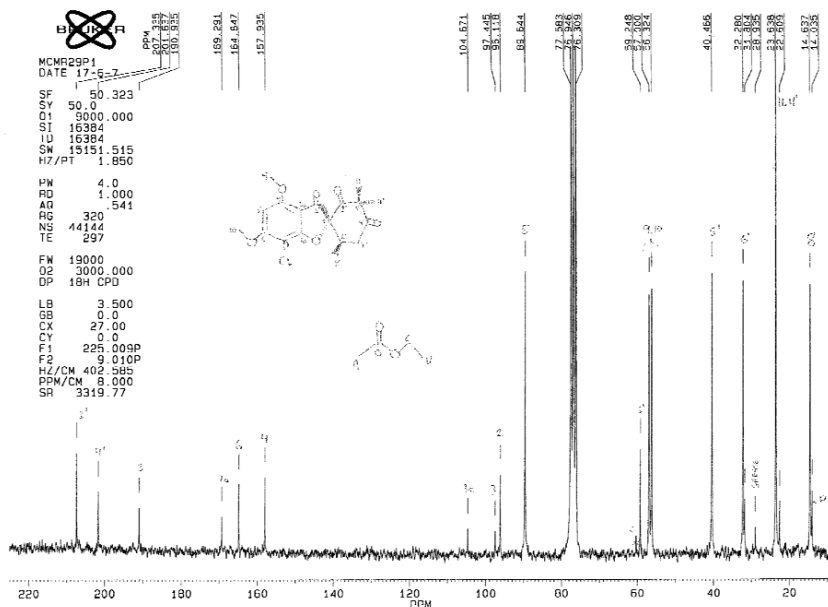
¹³C NMR (75 MHz, CDCl₃)



¹H NMR (300 MHz, CDCl₃) ^{13}C NMR (75 MHz, CDCl_3)

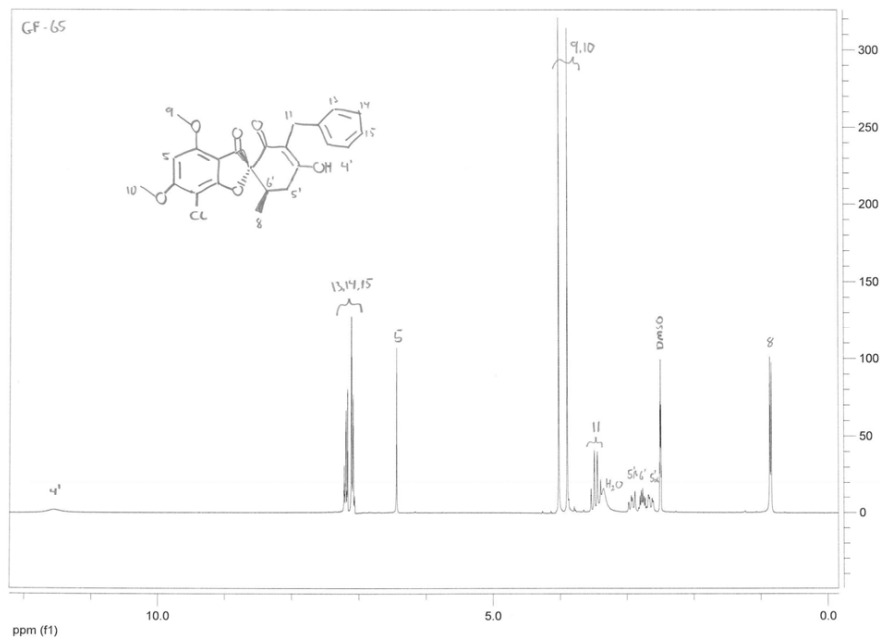
(2*S*,6'*R*,*E*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-ene-4'-one-4'-dimethylhydrazine) **30**

¹H NMR (300 MHz, CDCl₃) ^{13}C NMR (75 MHz, CDCl_3)

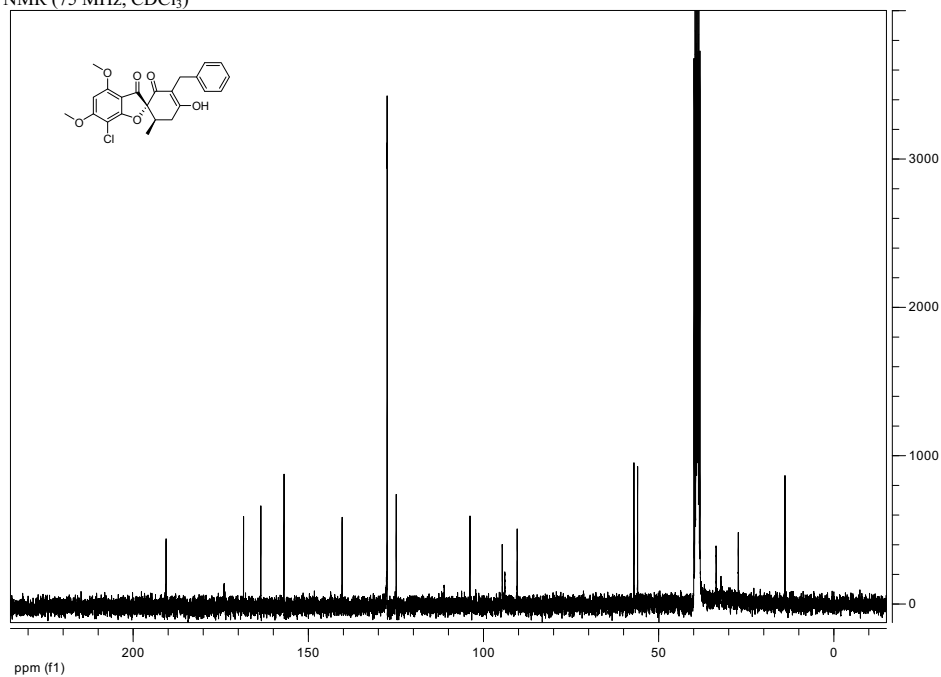
¹H NMR (500 MHz, CDCl₃) ^{13}C NMR (75 MHz, CDCl_3)

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(3'-benzyl-6'-methyl-cyclohex-2',4'-dione) **32**

^1H NMR (300 MHz, CDCl_3)

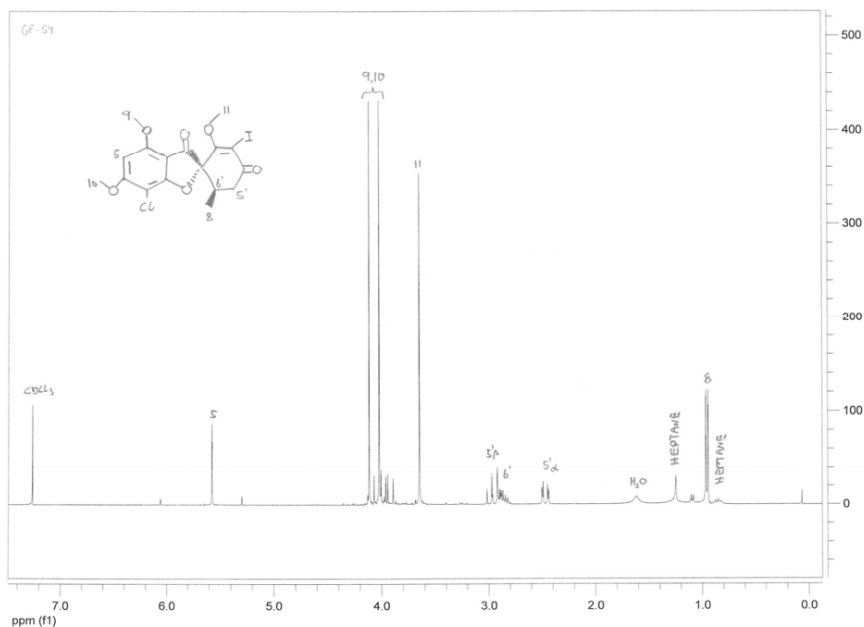


^{13}C NMR (75 MHz, CDCl_3)

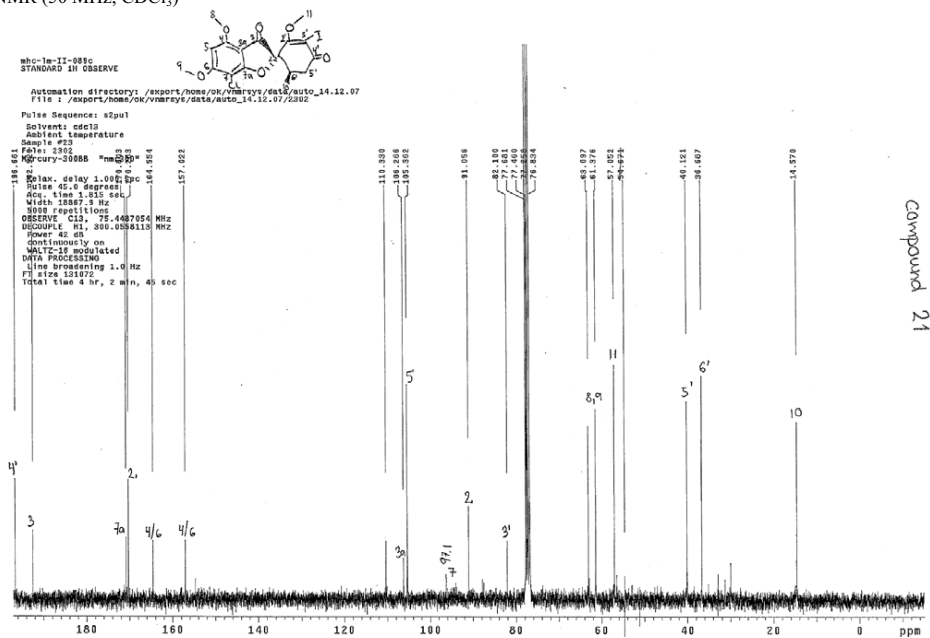


(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(3'-iodo-2'-methoxy-6'-methyl-cyclohex-2'-ene-4'-one) **33**

^1H NMR (300 MHz CDCl_3)

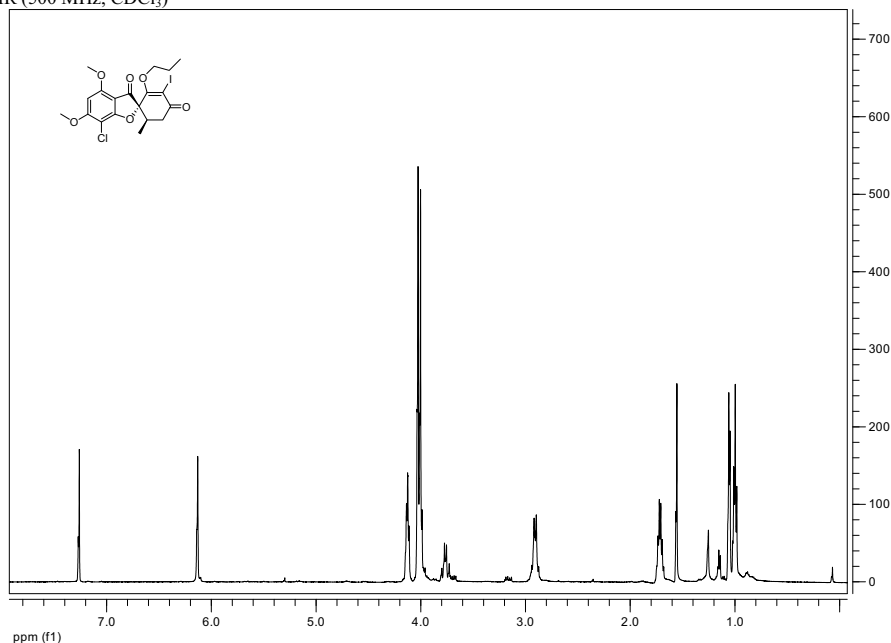


^{13}C NMR (50 MHz, CDCl_3)

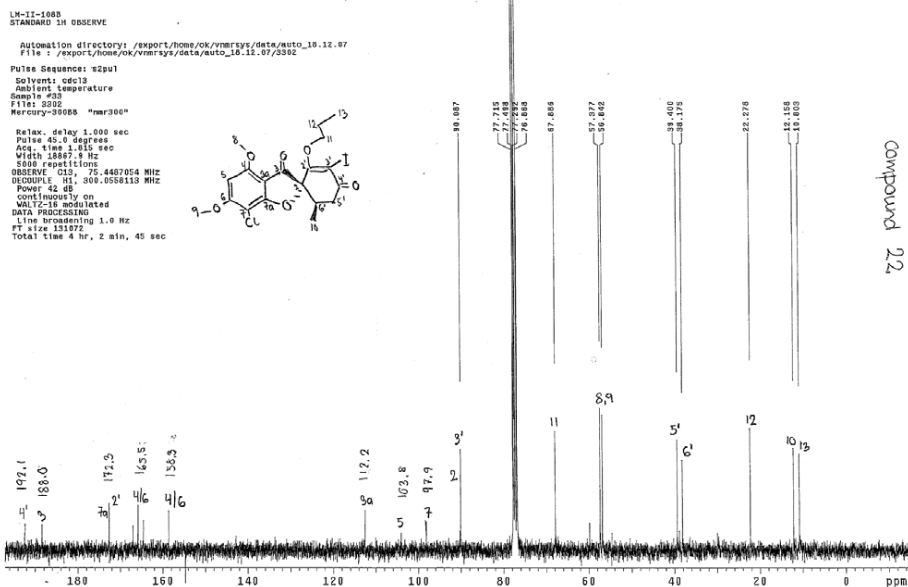


(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(3'-iodo-6'-methyl-2'-propoxy-cyclohex-2'-ene-4'-one) **34**

^1H NMR (500 MHz, CDCl_3)

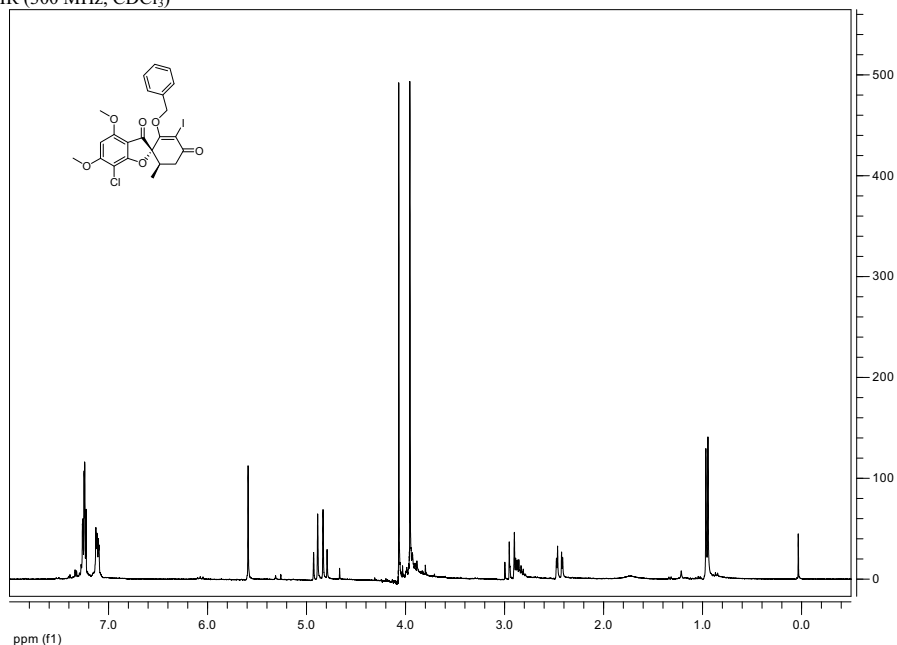


^{13}C NMR (75 MHz, CDCl_3)

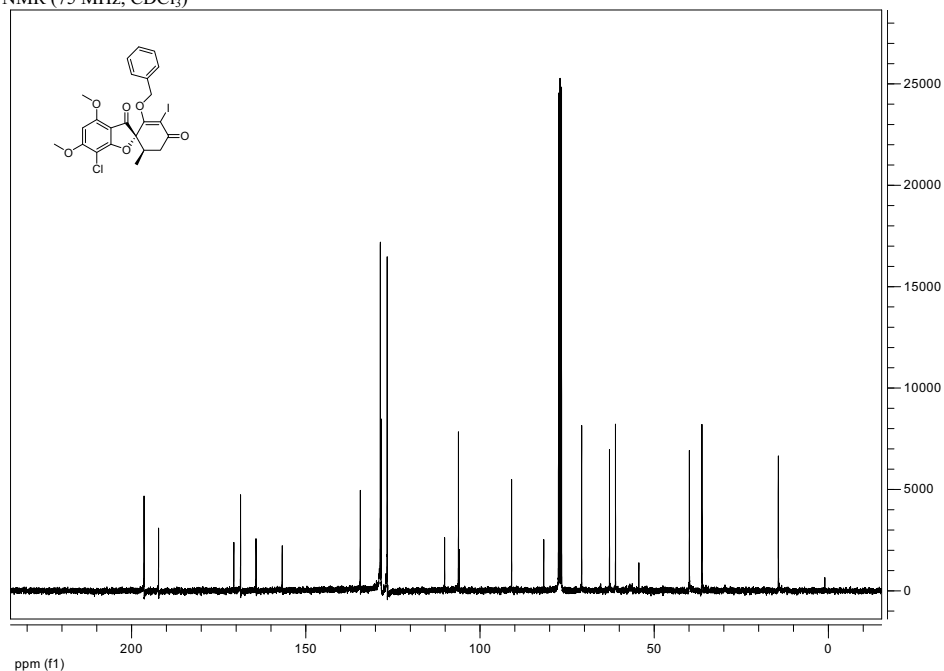


(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(2'-benzyloxy-3'-iodo-6'-methyl-cyclohex-2'-ene-4'-one) **35**

^1H NMR (300 MHz, CDCl_3)



^{13}C NMR (75 MHz, CDCl_3)



Supporting Information - Chapter 4

Disparate SAR Data of Griseofulvin Analogs for the Dermatophytes *Trichophyton mentagrophytes*, *T. rubrum* and MDA-MB-231 Cancer Cells

Mads H. Rønneest, Marc S. Raab, Simon Anderhub, Sven Boesen, Alwin Krämer, Thomas O. Larsen,^{*} Mads H. Clausen^{*}

Supporting Information

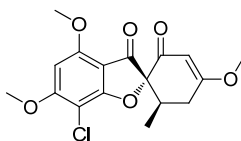
S2-S3	Experimental for compounds 3 , 39-41 and 44 .
S4-S27	NMR spectra of 3 , 7 , 8 , 15 , 19 , 21 , 22 , 26 , 28-35 and 38-44 .
S28-S41	UPLC-DAD chromatograms of all tested compounds.
S42	Figure S1 and S2.
S43	References.

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(6'-methyl-cyclohex-2',4'-dione) 2

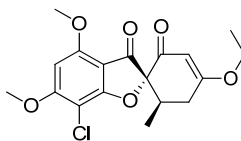
Griseofulvic acid (**2**) (3.0 mmol, 1 equiv.) was mixed with LiCl (15 mmol, 5 equiv.) and added to a solution of POCl₃ (15 mmol, 5 equiv.) in dioxane (14 mL). The mixture was stirred at 100 °C for 30 min, cooled to 0 °C and sat. aq. Na₂CO₃ was added carefully to the solution until slightly basic (pH 7-8). The aqueous phase was extracted with CH₂Cl₂ (3×20 mL) and the combined organic phases were dried (MgSO₄) and concentrated. The residue was purified by column chromatography (toluene:CH₂Cl₂:EtOAc 35:35:1) affording the desired products **15** and **16**.

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-one)-2-spiro-1'-(4'-chloro-6'-methyl-cyclohex-3'-en-2'-one) 15

Yield: 588 mg (55 %) (white crystals); *R_f*-value (EtOAc/heptane, 5:1): 0.63; m.p. 241-243 °C (Litt.¹); IR (neat, cm⁻¹): 1699, 1677, 1614, 1596, 1581 (Litt.¹); ¹H NMR (CDCl₃, 300 MHz): δ 6.28 (1H, d, *J* = 2.2 Hz), 6.10 (1H, s), 4.01 (3H, s), 3.94 (3H, s), 3.40 (1H, ddd, *J* = 18.5, 11.2, 2.2 Hz), 2.93 (1H, ddq, *J* = 11.2, 5.7, 6.7 Hz), 2.75 (1H, dd, *J* = 18.5, 5.7 Hz), 1.04 (3H, d, *J* = 6.7 Hz); ¹³C NMR (CDCl₃, 50 MHz): δ 190.3, 187.0, 169.5, 164.6, 160.0, 157.8, 125.3, 104.7, 97.5, 94.6, 89.6, 57.0, 56.3, 38.6, 36.5, 14.2; [α]_D²⁰ = +269° (c = 0.5 in CHCl₃); HRMS (ESI⁺) calcd for M+H [C₁₆H₁₄Cl₂O₅]⁺ 357.0297, found 357.0304; Anal. Calcd for C₁₆H₁₄Cl₂O₅: C, 53.80; H, 3.95. Found: C, 53.69; H, 3.88.

General Procedure for the Synthesis of Enol Ethers by Solvolysis (39-41 and 44).**(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(4'-methoxy-6'-methyl-cyclohex-3'-en-2'-one) 3**

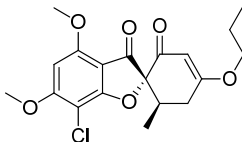
Yield: 116 mg (22 %) (white crystals); *R_f*-value (EtOAc/heptane, 5:1): 0.48; m.p. 197-198 °C (Litt.⁵); IR (neat, cm⁻¹): 1701, 1657, 1607, 1589; ¹H NMR (CDCl₃, 300 MHz): δ 6.07 (1H, s), 5.44 (1H, s, *J* = 1.5 Hz), 4.00 (3H, s), 3.91 (3H, s), 3.76 (3H, s), 3.18 (1H, ddd, *J* = 17.5, 12.0, 1.5 Hz), 2.83 (1H, m), 2.46 (1H, dd, *J* = 17.5, 5.7 Hz), 1.01 (3H, d, *J* = 6.7 Hz) (Litt.⁵); ¹³C NMR (CDCl₃, 50 MHz): δ 191.6, 188.6, 178.8, 169.7, 164.3, 157.6, 105.1, 99.6, 97.3, 95.1, 89.4, 56.9, 56.2 (2C), 35.1, 32.6, 14.4; [α]_D²⁰ = +142° (c = 0.3 in CHCl₃); HRMS (ESI⁺) calcd for [M+H]⁺ [C₁₇H₁₈ClO₆]⁺ 353.0792, found 353.0790; Anal. Calcd for C₁₇H₁₇ClO₆: C, 57.88; H, 4.86. Found: C, 57.75; H, 4.60.

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(4'-ethoxy-6'-methyl-cyclohex-3'-en-2'-one) 39

Yield: 145 mg (64 %) (white crystals); *R_f*-value (EtOAc/heptane, 5:1): 0.67; m.p. 167-168 °C (Litt.²); IR (neat, cm⁻¹): 1703, 1657, 1609, 1589 (Litt.³); ¹H NMR (CDCl₃, 300 MHz): δ 6.08 (1H, s), 5.41 (1H, d, *J* = 1.2 Hz), 4.00 (3H, s), 3.98 (2H, q, *J* = 7.0 Hz), 3.92 (3H, s), 3.18 (1H, ddd, *J* = 17.6, 12.1, 1.2 Hz), 2.84 (1H, ddq, *J* = 12.1, 5.7, 6.7 Hz), 2.45 (1H, dd, *J* = 17.6, 5.7 Hz), 1.39 (3H, t, *J* = 7.0 Hz), 1.02 (3H, d, *J* = 6.7 Hz); ¹³C NMR (CDCl₃, 50 MHz): δ 191.7, 188.8, 178.0, 169.6, 164.3, 157.6, 105.1, 99.9, 97.2, 95.0, 89.4, 64.9,

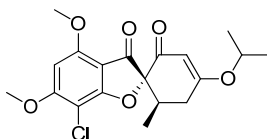
56.9, 56.2, 35.1, 33.2, 14.4, 14.0; $[\alpha]_D^{20} = +198^\circ$ ($c = 0.4$ in CHCl_3); Anal. Calcd for $\text{C}_{18}\text{H}_{19}\text{ClO}_6$: C, 58.94; H, 5.22. Found: C, 58.78; H, 5.20.

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(4'-propoxy-6'-methyl-cyclohex-3'-en-2'-one) **40**



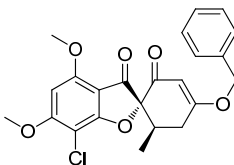
Yield: 146 mg (63%) (white crystals); R_f -value (EtOAc/heptane, 5:1): 0.75; m.p. 176-178 $^\circ\text{C}$ (Litt.⁴); IR (neat, cm^{-1}): 1703, 1657, 1608, 1590 (Litt.³); ^1H NMR (CDCl_3 , 500 MHz): δ 6.08 (1H, s), 5.41 (1H, s), 4.00 (3H, s), 3.92 (3H, s), 3.91-3.82 (2H, m), 3.19 (1H, dd, $J = 17.6, 12.1$ Hz), 2.84 (1H, ddq, $J = 12.1, 6.7, 5.7$ Hz), 2.46 (1H, dd, $J = 17.6, 5.7$ Hz), 1.83-1.74 (2H, m), 1.02 (3H, d, $J = 6.7$ Hz), 0.99 (3H, t, $J = 7.4$ Hz); ^{13}C NMR (CDCl_3 , 50 MHz): δ 191.7, 188.8, 178.2, 169.6, 164.4, 157.6, 105.1, 99.9, 97.2, 95.0, 89.4, 70.7, 56.9, 56.2, 35.1, 33.1, 21.7, 14.4, 10.2; $[\alpha]_D^{20} = +192^\circ$ ($c = 0.5$ in CHCl_3); Anal. Calcd for $\text{C}_{19}\text{H}_{21}\text{ClO}_6$: C, 59.92; H, 5.56. Found: C, 59.86; H, 5.47

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(4'-isopropoxy-6'-methyl-cyclohex-3'-en-2'-one) **41**

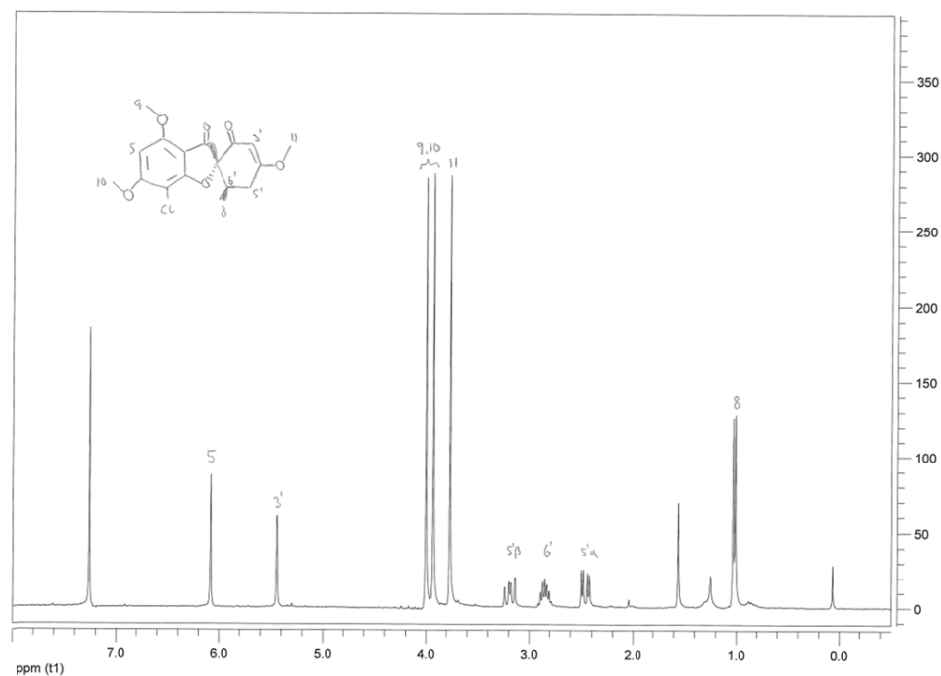
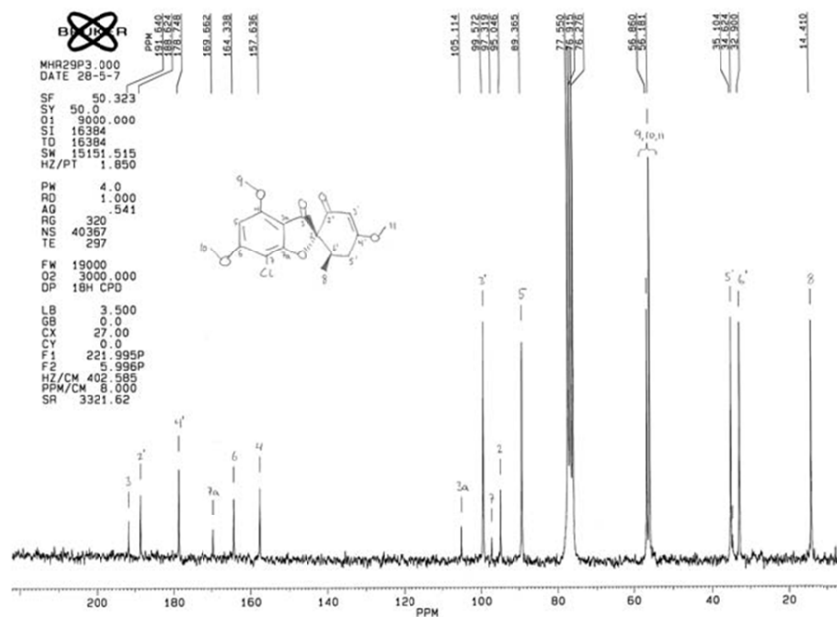


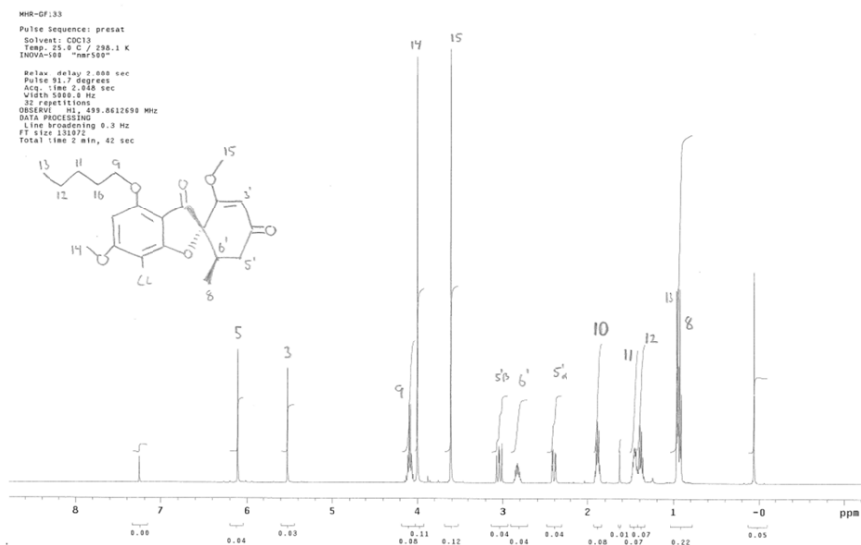
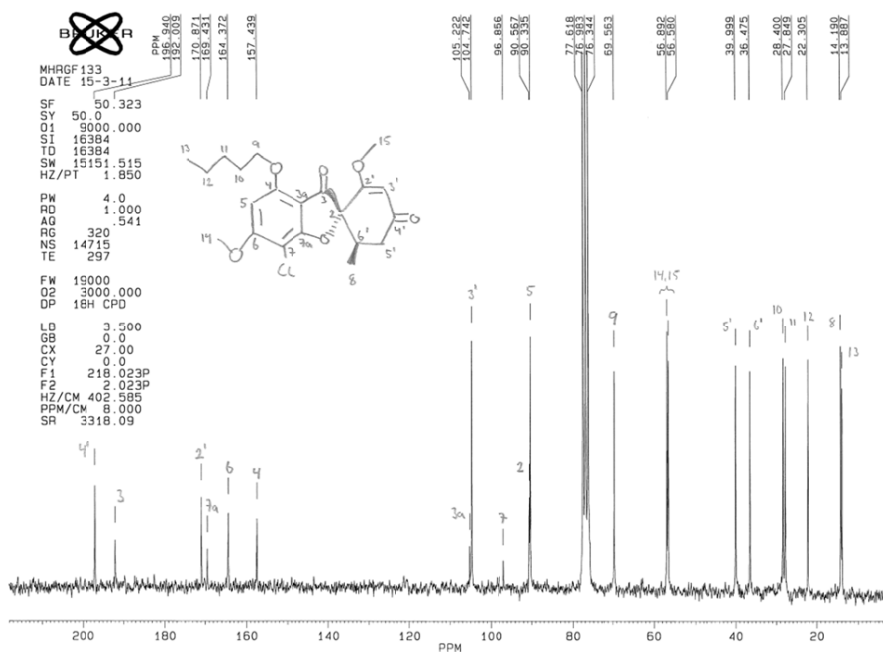
Yield: 109 mg (47%) (white crystals); R_f -value (EtOAc/heptane, 5:1): 0.71; m.p. 186-187 $^\circ\text{C}$ (Litt.⁴); IR (neat, cm^{-1}): 1704, 1657, 1611, 1589 (Litt.³); ^1H NMR (CDCl_3 , 300 MHz): δ 6.08 (1H, s), 5.41 (1H, s), 4.49 (1H, sept., $J = 6.1$ Hz), 4.00 (3H, s), 3.92 (3H, s), 3.15 (1H, ddd, $J = 17.6, 12.1, 1.2$ Hz), 2.83 (1H, ddq, $J = 12.1, 6.7, 5.7$ Hz), 2.42 (1H, dd, $J = 17.6, 5.7$ Hz), 1.33 (6H, d, $J = 6.1$ Hz), 1.01 (3H, d, $J = 6.7$ Hz); ^{13}C NMR (CDCl_3 , 50 MHz): δ 191.8, 188.8, 177.0, 169.7, 164.3, 157.6, 105.2, 100.2, 97.4, 95.1, 89.3, 71.9, 56.9, 56.2, 35.0, 33.6, 21.5, 21.2, 14.4; $[\alpha]_D^{20} = +153^\circ$ ($c = 0.4$ in CHCl_3); Anal. Calcd for $\text{C}_{19}\text{H}_{21}\text{ClO}_6$: C, 59.92; H, 5.56. Found: C, 59.84; H, 5.50.

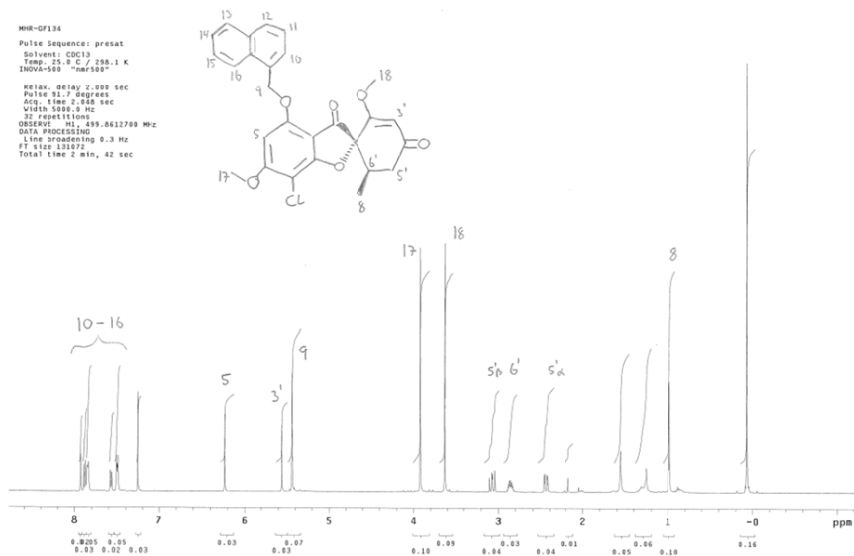
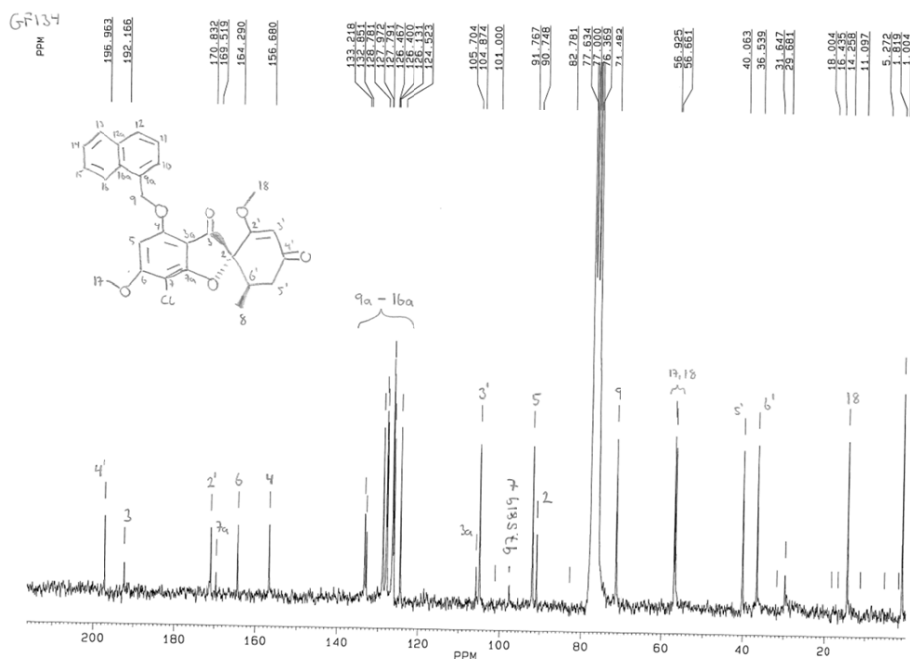
(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(4'-benzyloxy-6'-methyl-cyclohex-3'-en-2'-one) **44**

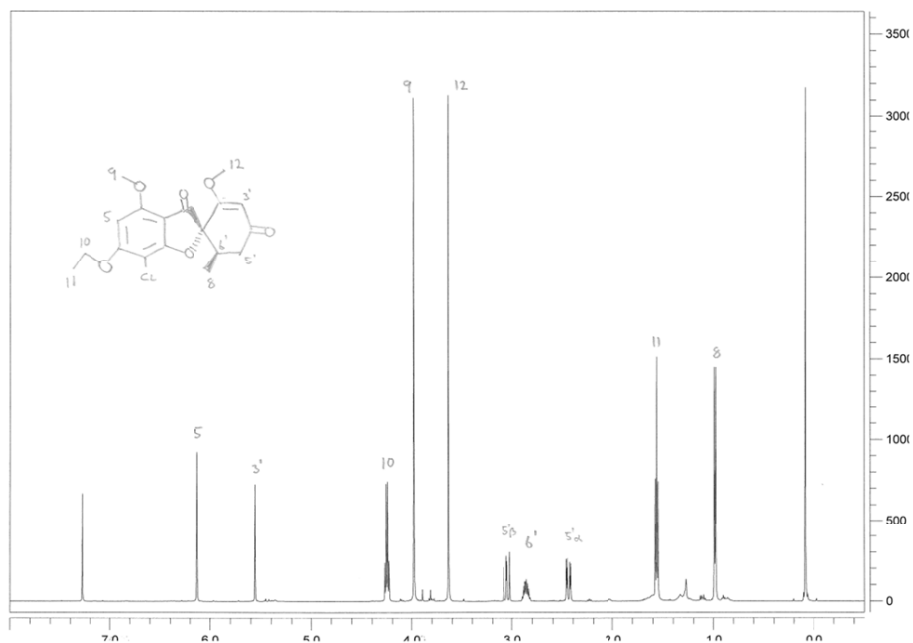
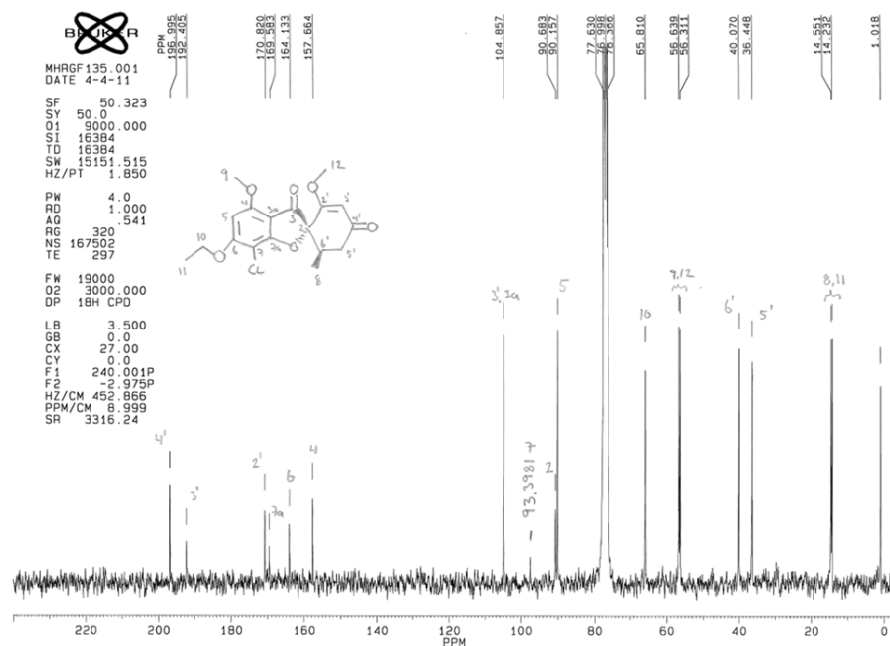


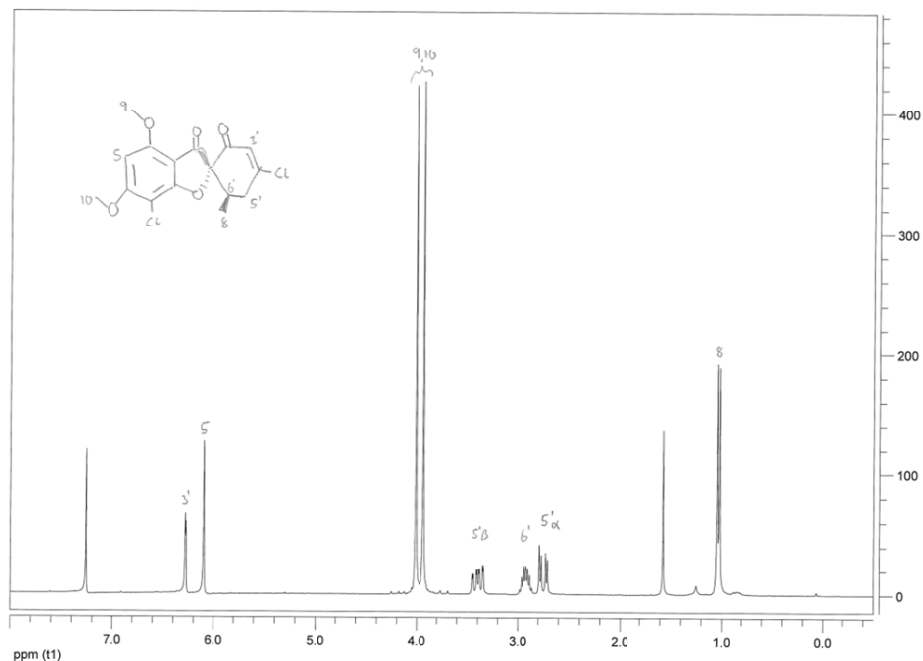
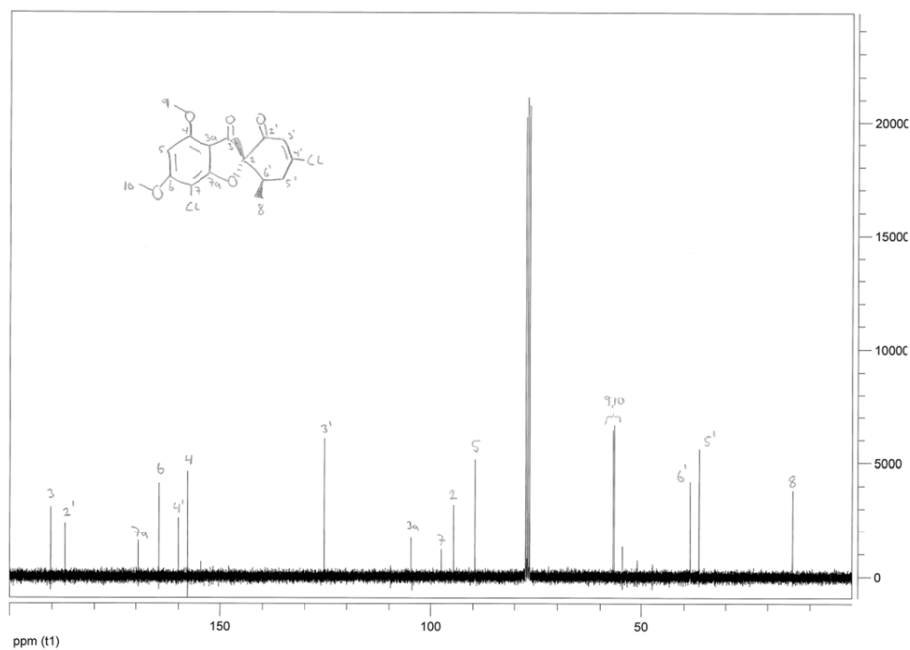
Yield: 398 mg (31%) (yellow oil); R_f -value (EtOAc/heptane, 5:1): 0.59; IR (neat, cm^{-1}): 1703, 1658, 1609, 1589; ^1H NMR (CDCl_3 , 300 MHz): δ 7.42-7.33 (5H, m), 6.06 (1H, s), 5.53 (1H, s), 4.96 (1H, d, $J = 17.2$ Hz), 4.94 (1H, d, $J = 17.2$ Hz), 3.99 (3H, s), 3.86 (3H, s), 3.25 (1H, dd, $J = 17.6, 12.0$ Hz), 2.90-2.81 (1H, m), 2.53 (1H, dd, $J = 17.6, 5.7$ Hz), 1.02 (3H, d, $J = 6.7$ Hz); ^{13}C NMR (CDCl_3 , 50 MHz): δ 191.6, 188.8, 177.7, 169.6, 164.4, 157.6, 134.4, 128.6 (2C), 128.1, 127.8 (2C), 104.9, 100.5, 97.1, 94.9, 89.4, 71.0, 56.9, 56.1, 35.1, 33.1, 14.4; $[\alpha]_D^{20} = +307^\circ$ ($c = 0.5$ in CHCl_3); HRMS (ESI⁺) calcd for $[\text{M}+\text{H}]^+$ $[\text{C}_{23}\text{H}_{22}\text{ClNO}_6]^+$ 429.1105, found 429.1107.

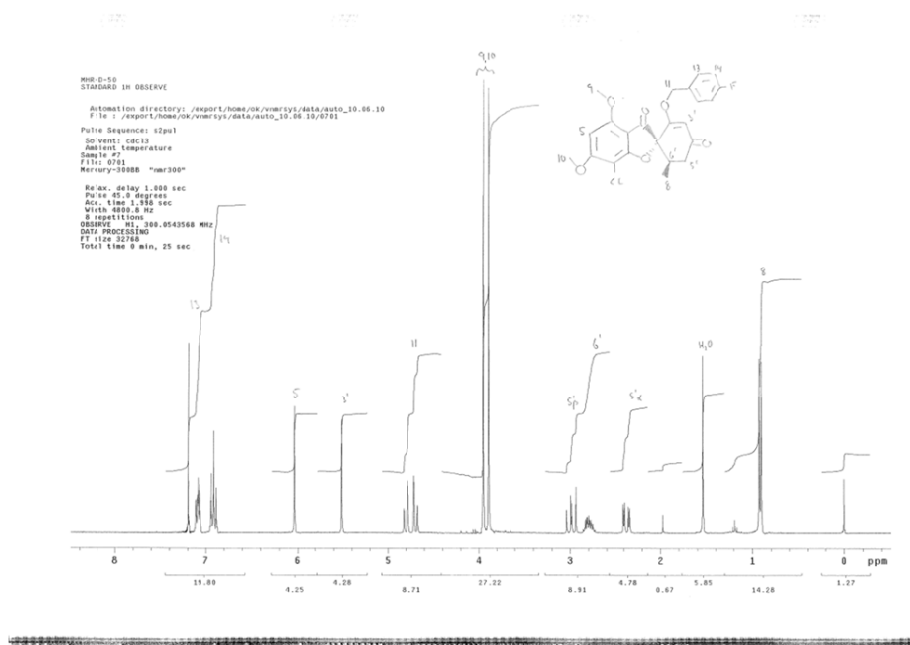
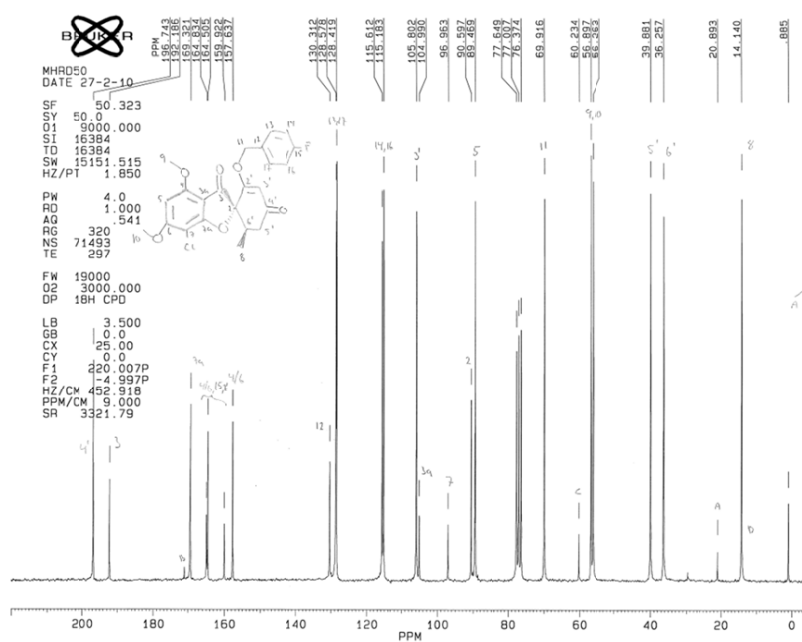
¹H NMR (300 MHz, CDCl₃) **3** ^{13}C NMR (50 MHz, CDCl_3) **3**

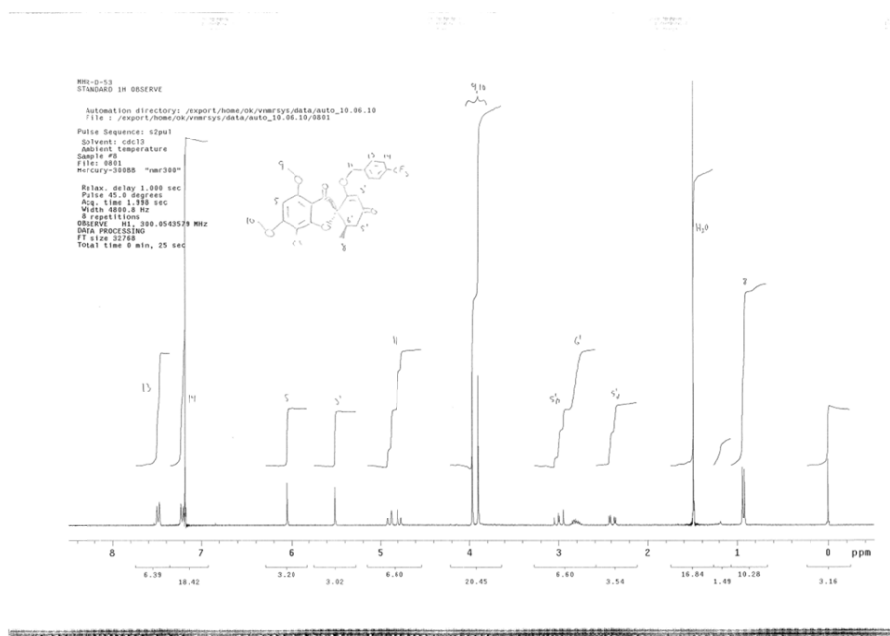
¹H NMR (300 MHz, CDCl₃) 7¹³C NMR (50 MHz, CDCl₃) 7

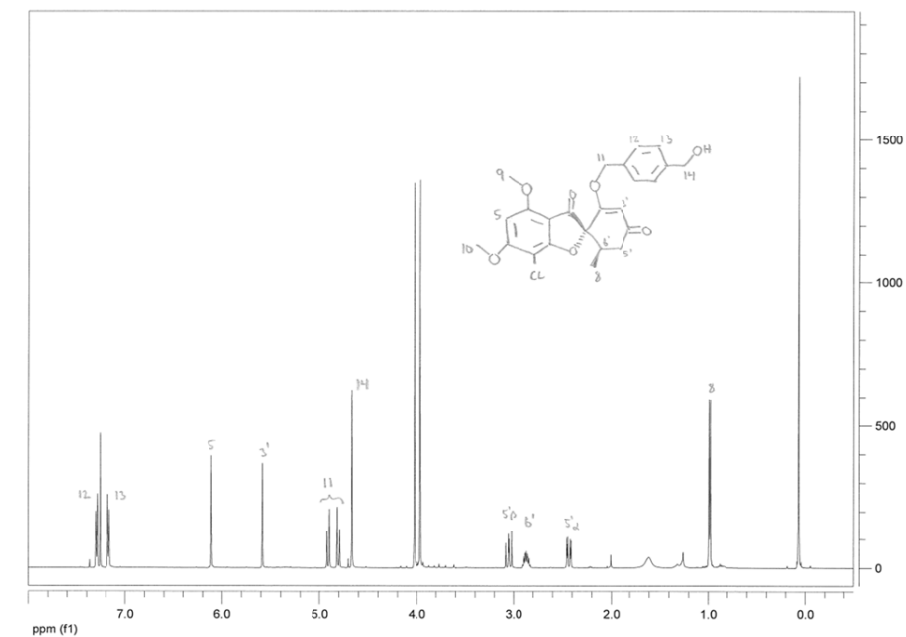
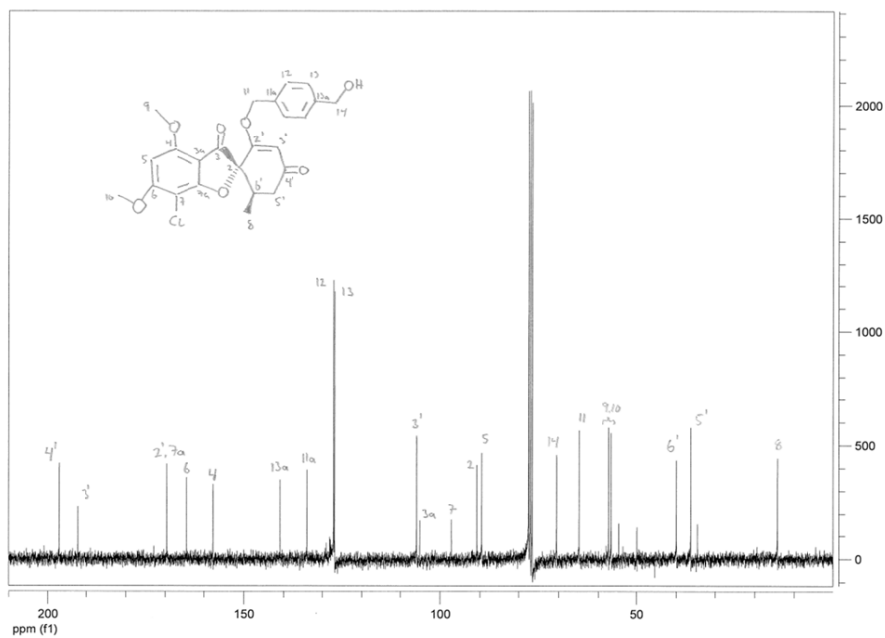
¹H NMR (300 MHz, CDCl₃) **8**¹³C NMR (50 MHz, CDCl₃) **8**

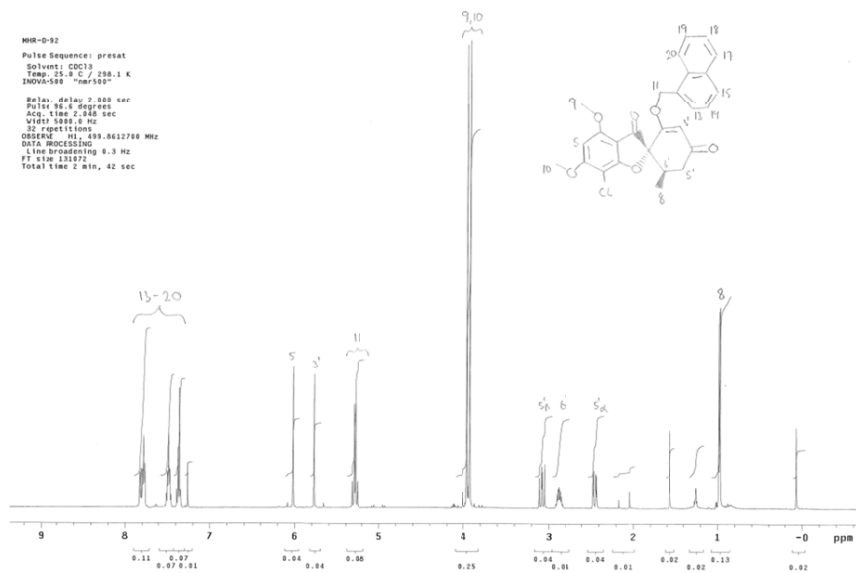
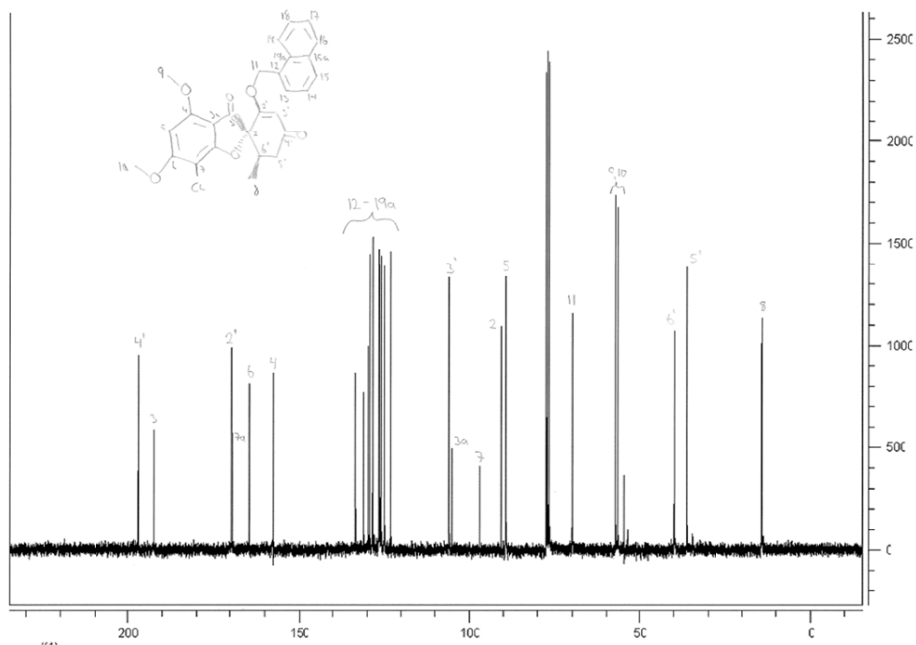
¹H NMR (300 MHz, CDCl₃) **14**¹³C NMR (50 MHz, CDCl₃) **14**

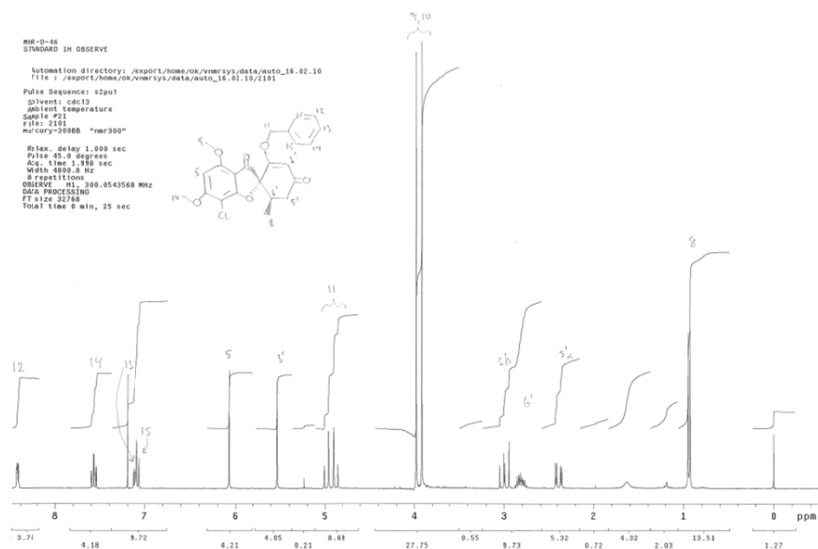
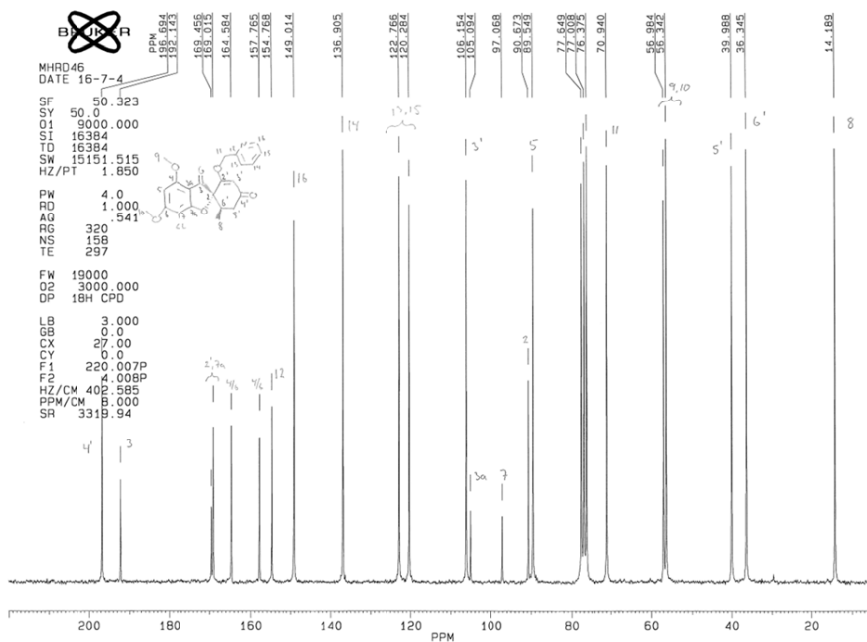
^1H NMR (300 MHz, CDCl_3) **15** ^{13}C NMR (75 MHz, CDCl_3) **15**

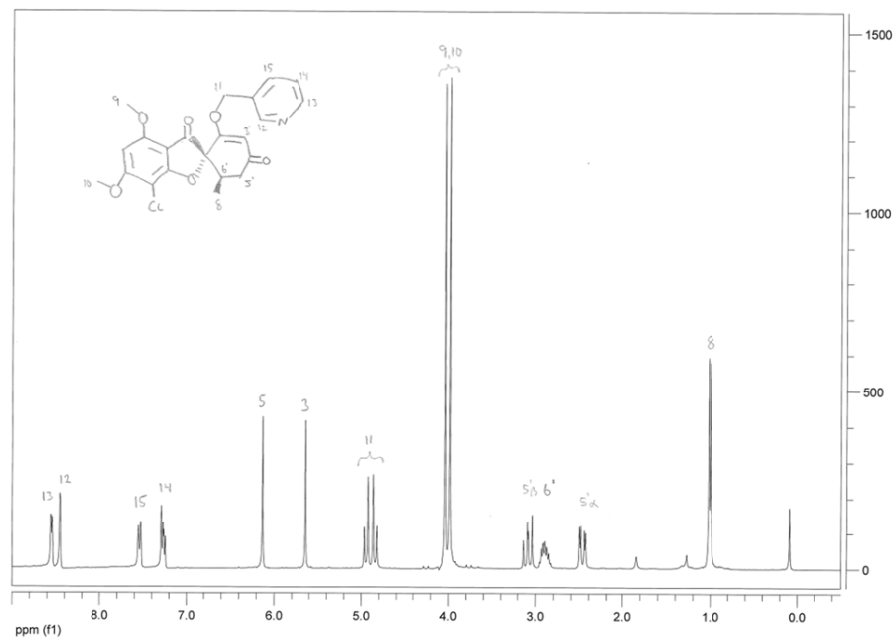
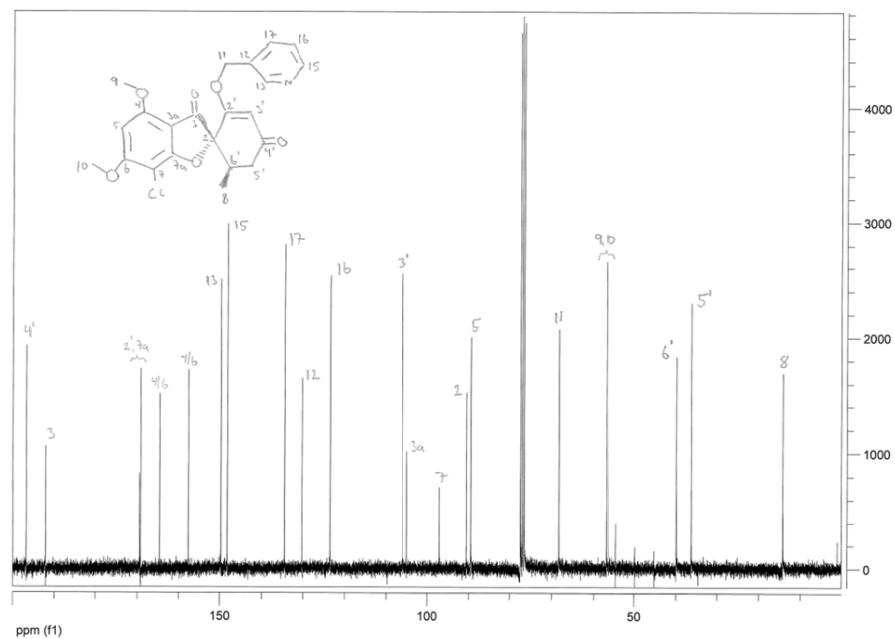
¹H NMR (300 MHz, CDCl₃) **19**¹³C NMR (50 MHz, CDCl₃) **19**

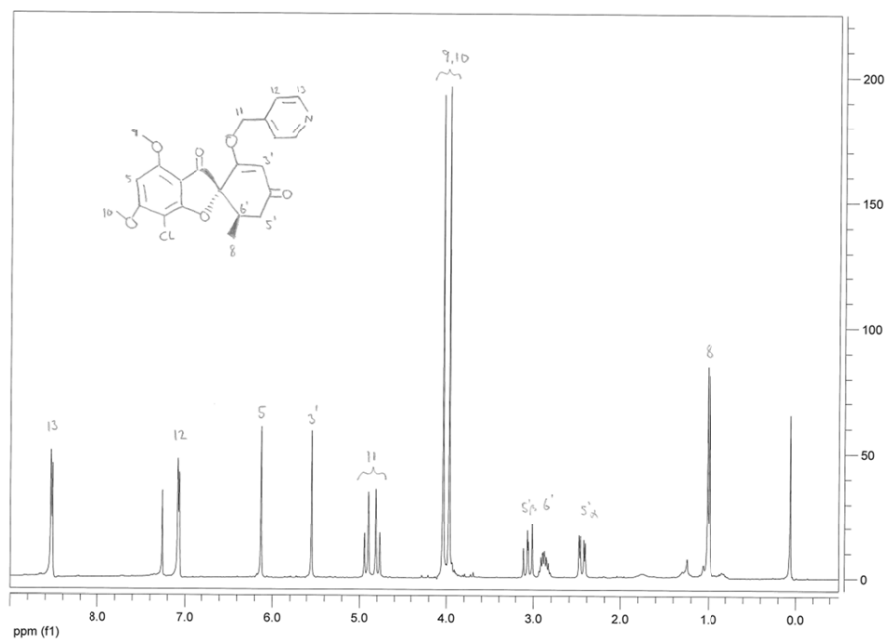
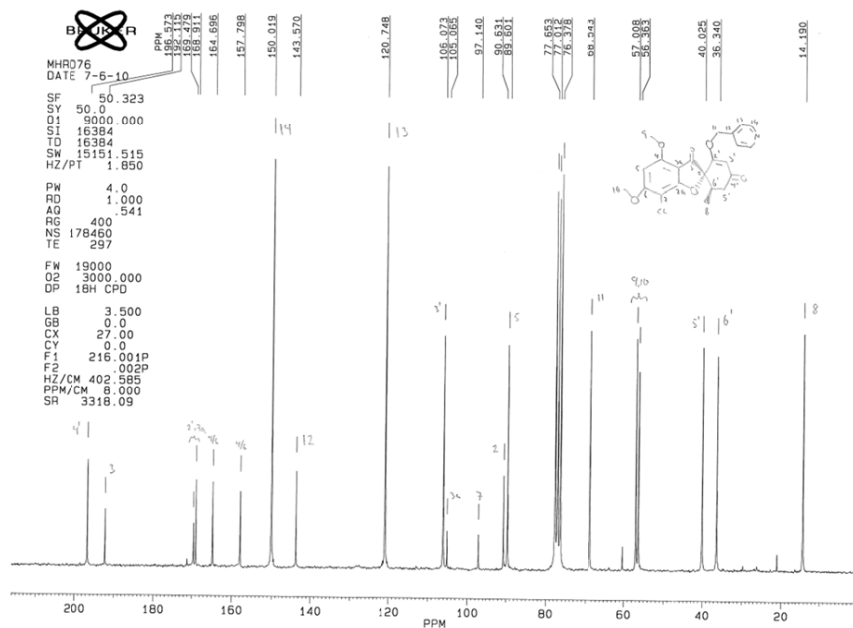
¹H NMR (300 MHz, CDCl₃) **21**

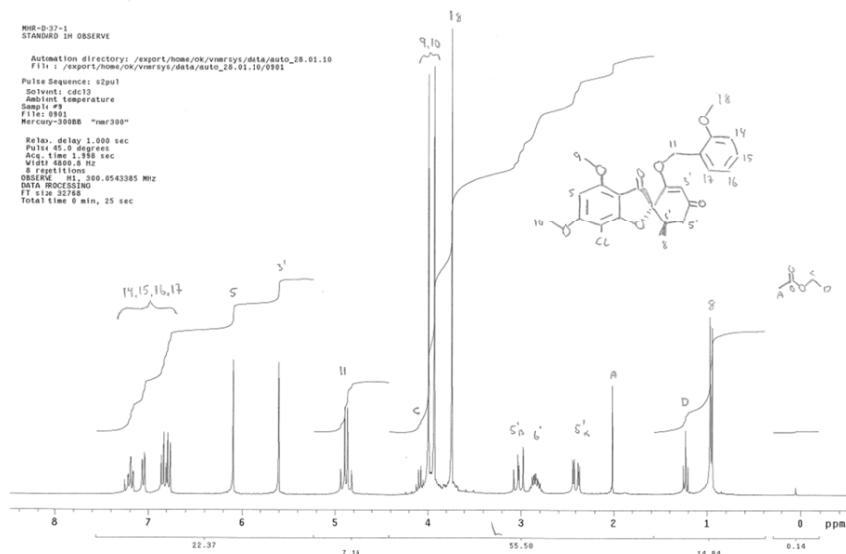
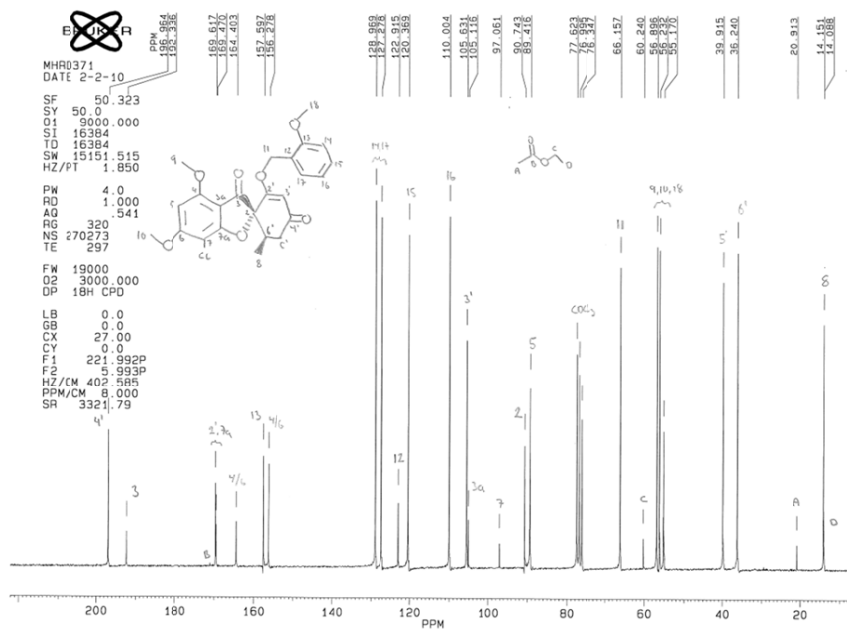
¹H NMR (300 MHz, CDCl₃) **22**¹³C NMR (50 MHz, CDCl₃) **22**

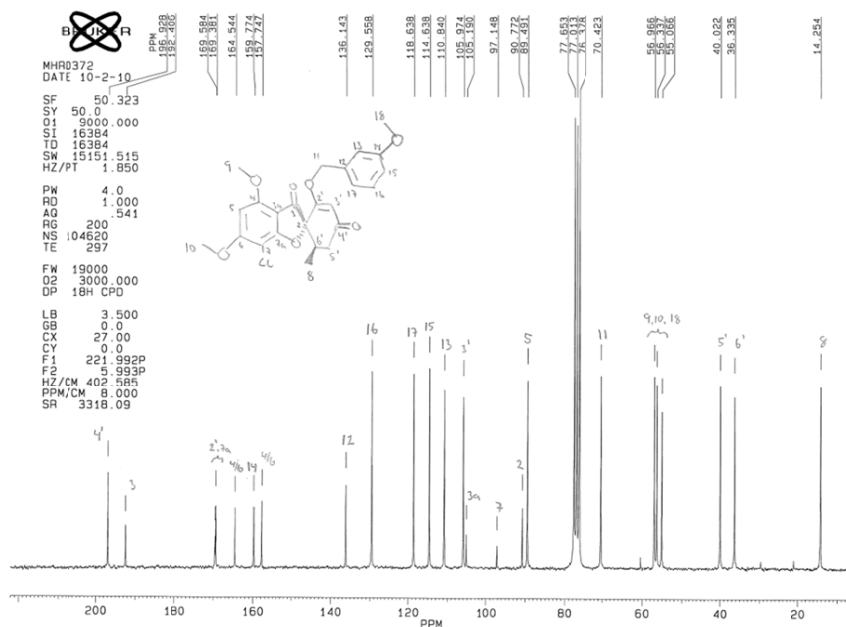
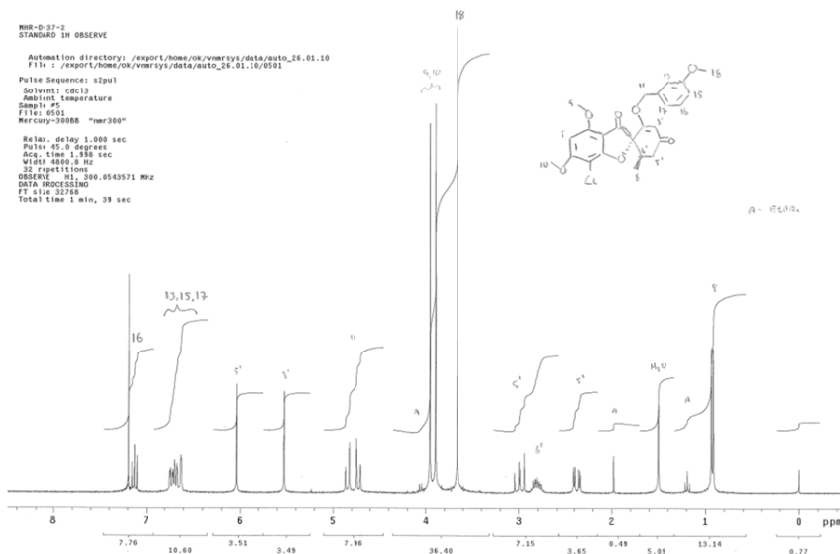
^1H NMR (500 MHz, CDCl_3) **26** ^{13}C NMR (75 MHz, CDCl_3) **26**

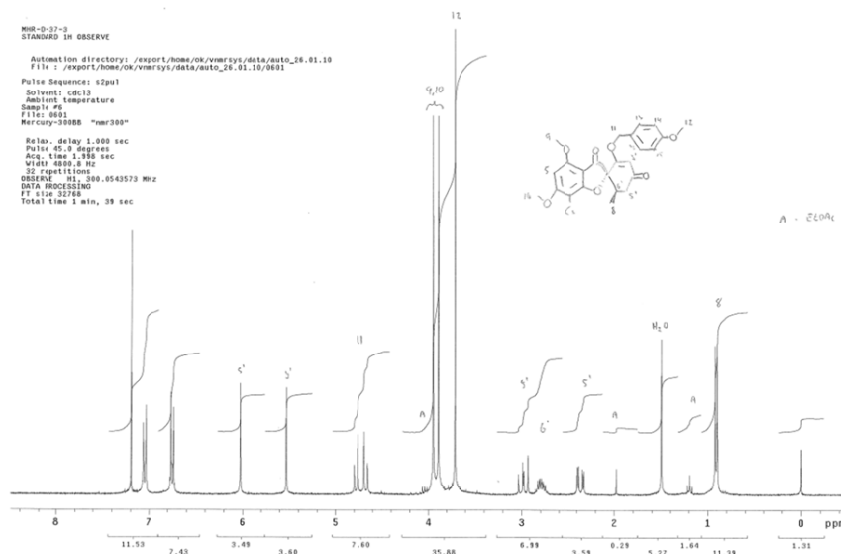
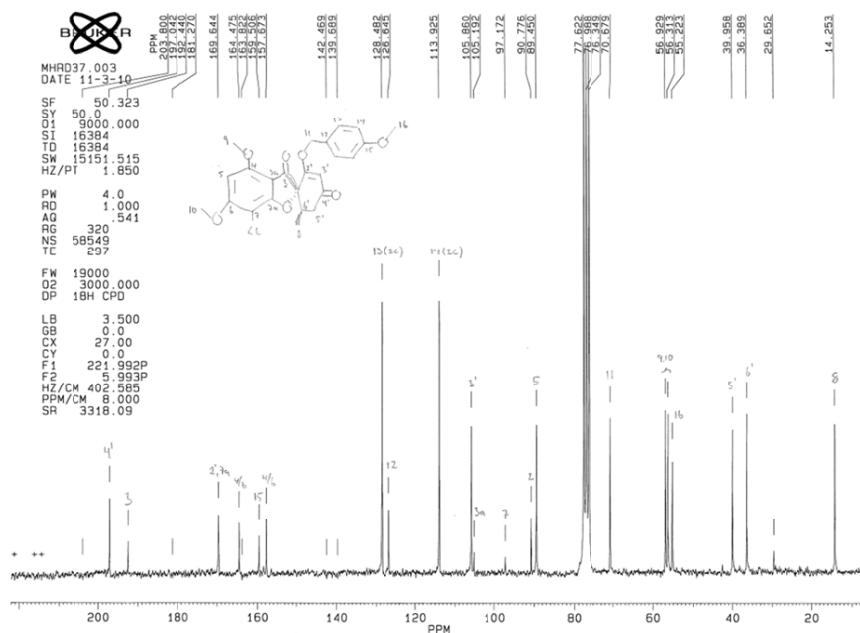
¹H NMR (300 MHz, CDCl₃) **28**¹³C NMR (50 MHz, CDCl₃) **28**

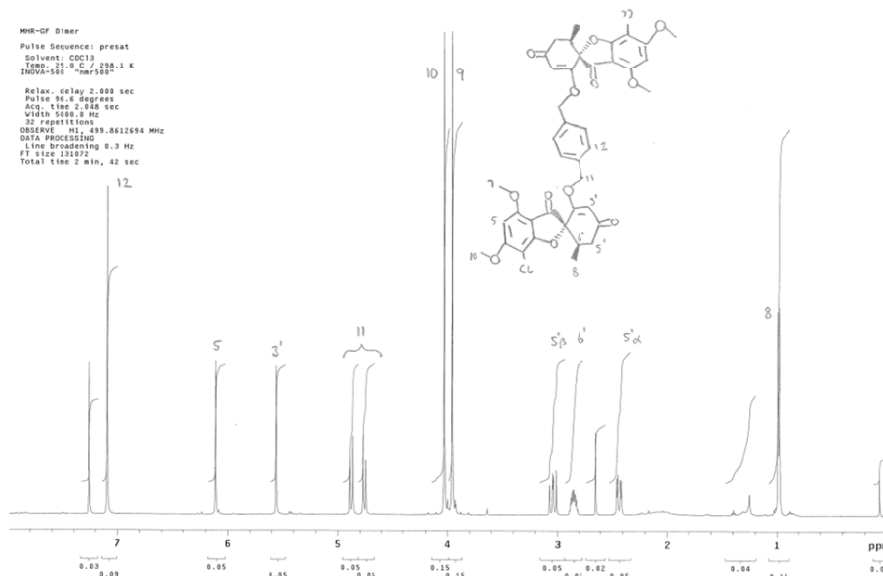
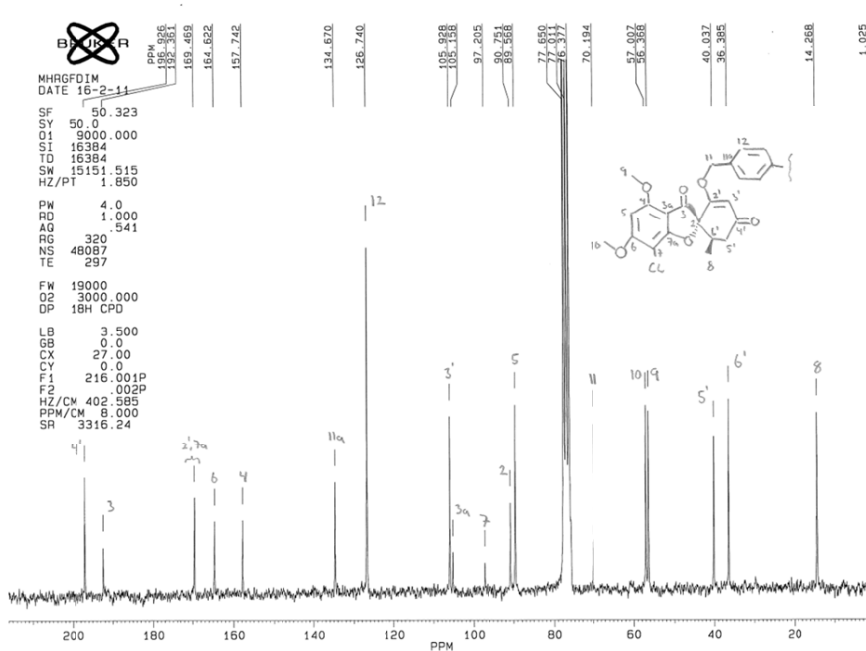
^1H NMR (300 MHz, CDCl_3) **29** ^{13}C NMR (75 MHz, CDCl_3) **29**

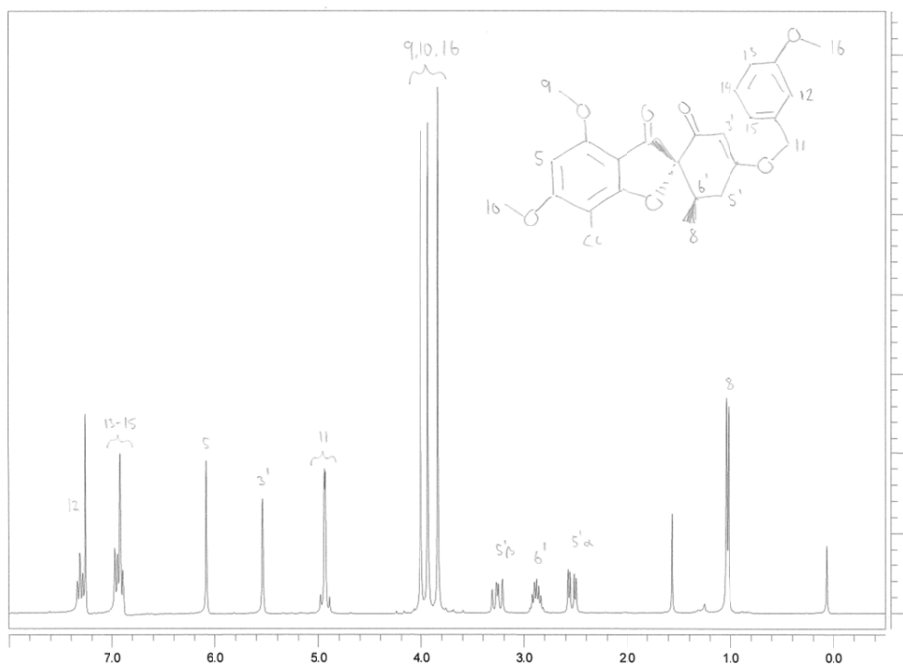
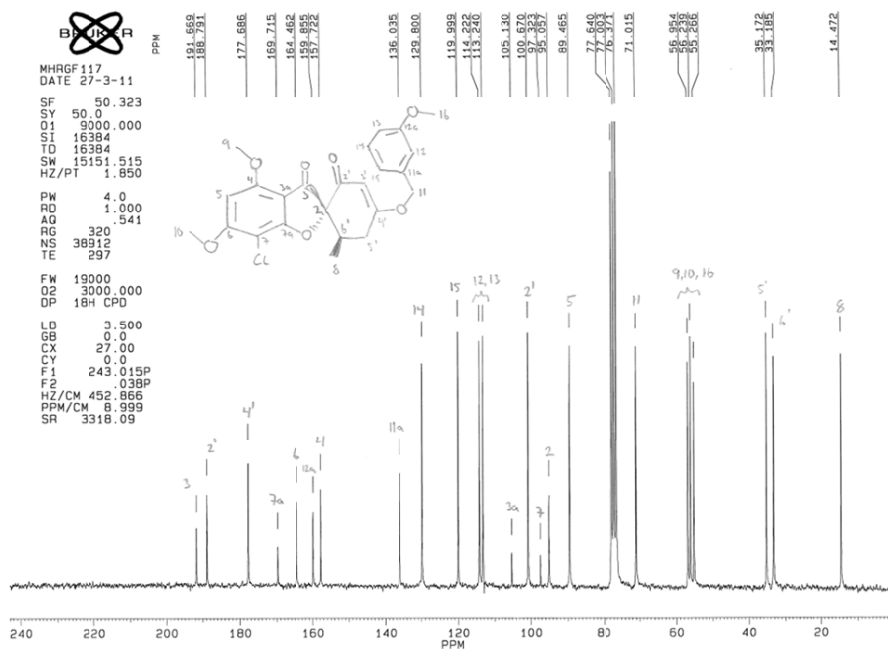
¹H NMR (300 MHz, CDCl₃) **30**¹³C NMR (50 MHz, CDCl₃) **30**

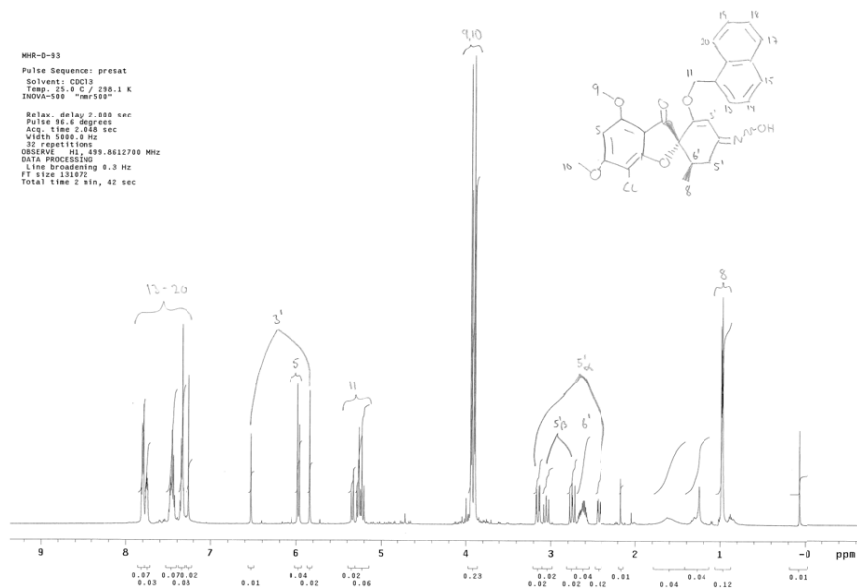
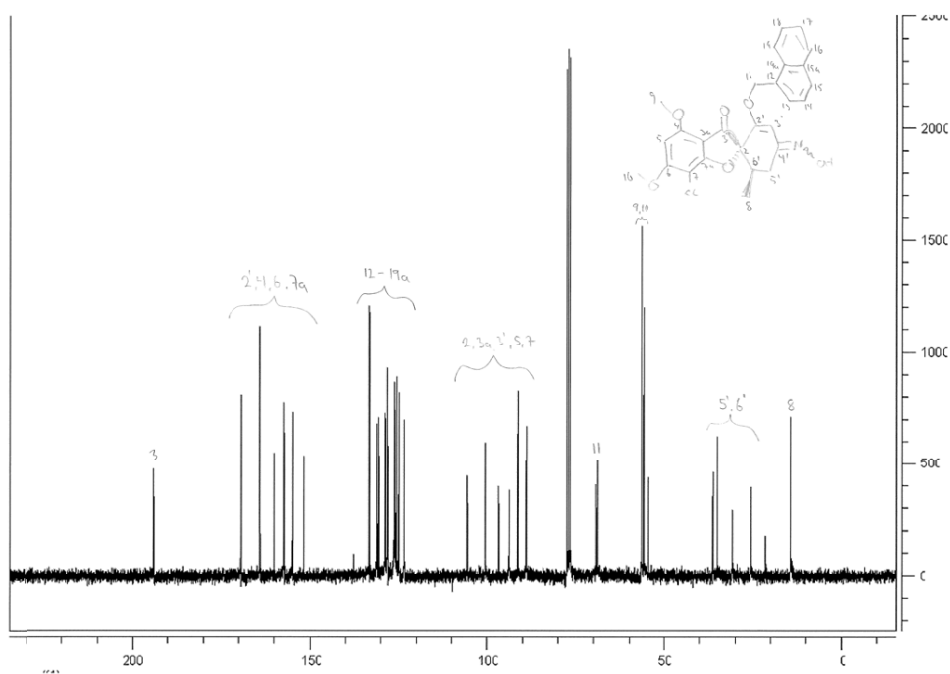
^1H NMR (300 MHz, CDCl_3) **31** ^{13}C NMR (50 MHz, CDCl_3) **31**

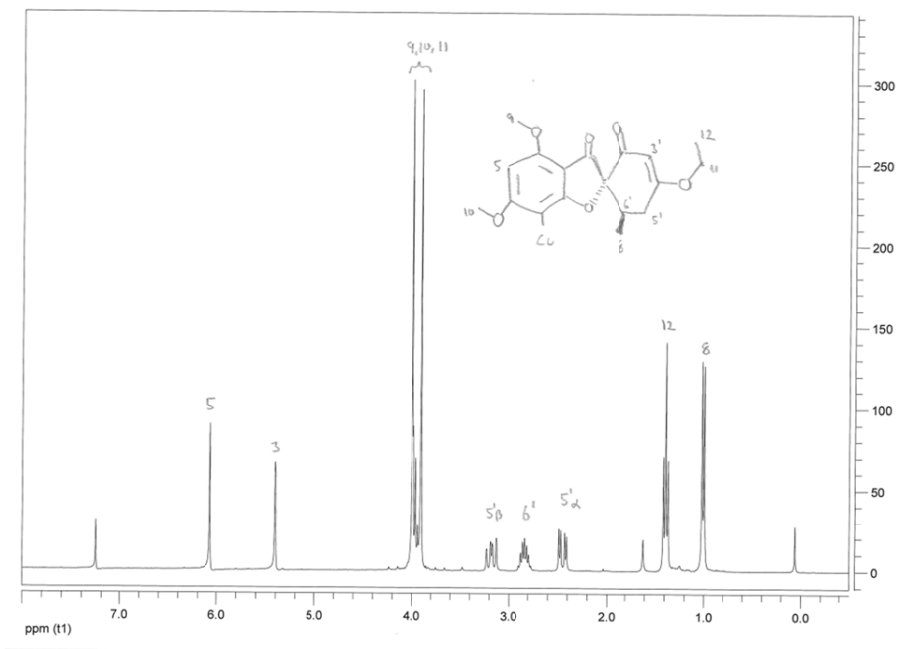
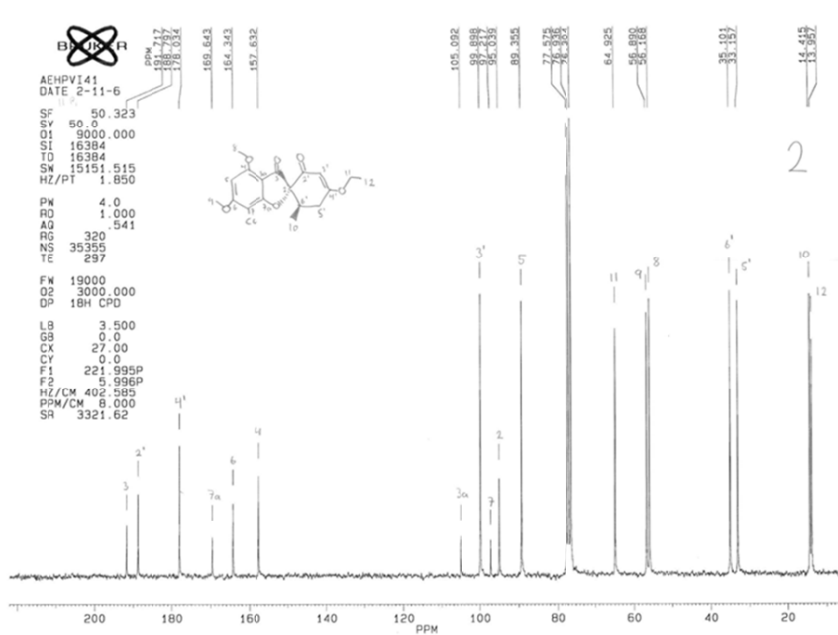


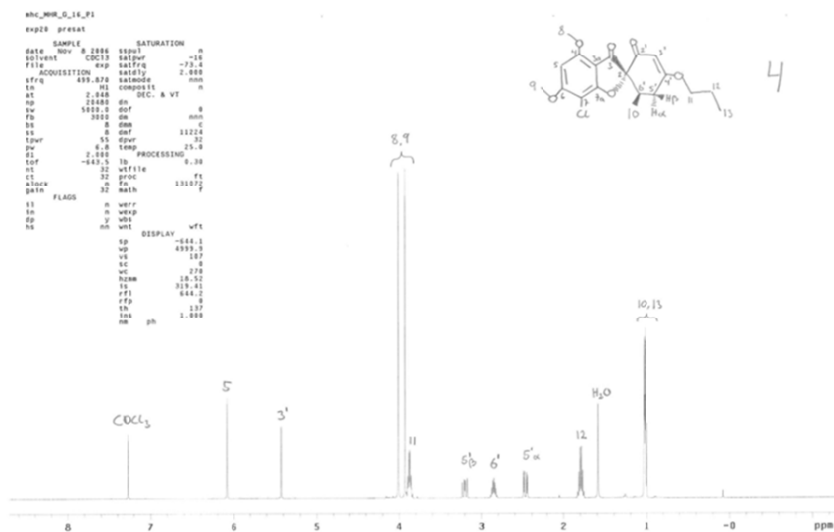
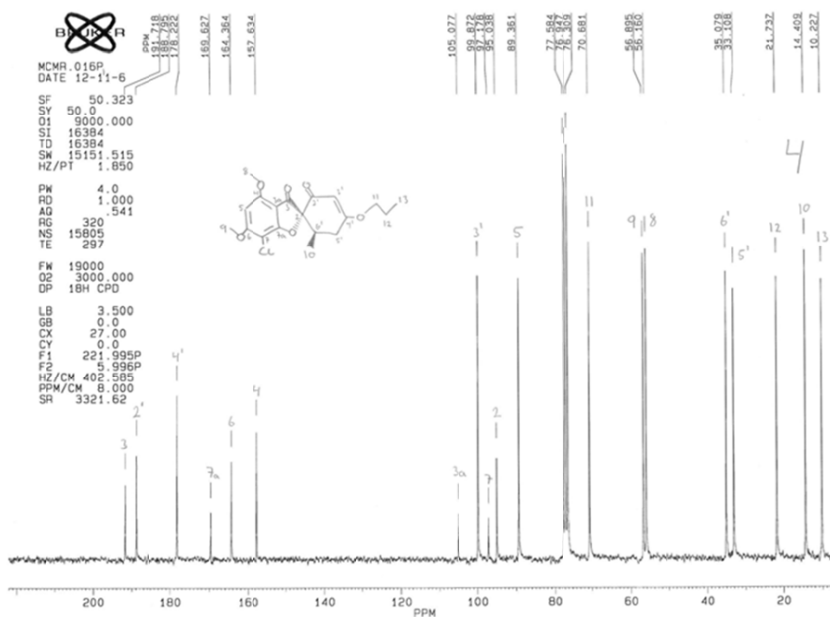
¹H NMR (300 MHz, CDCl₃) **33**¹³C NMR (50 MHz, CDCl₃) **33**

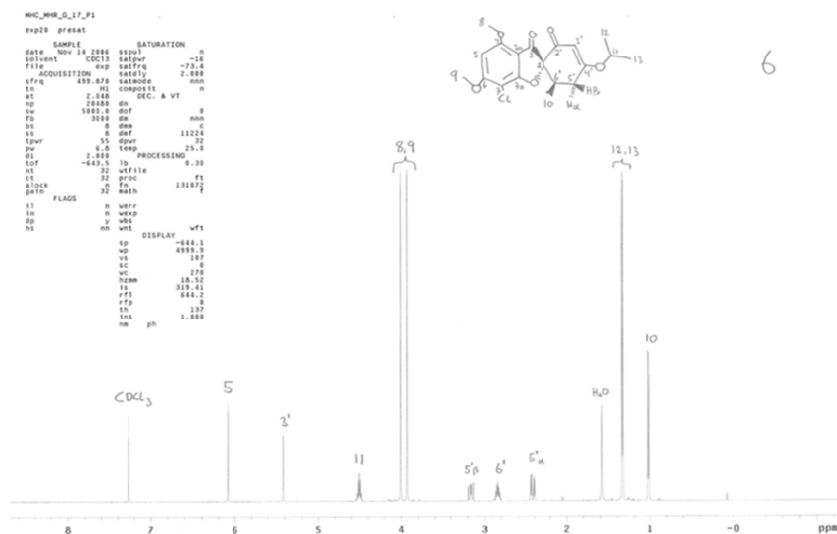
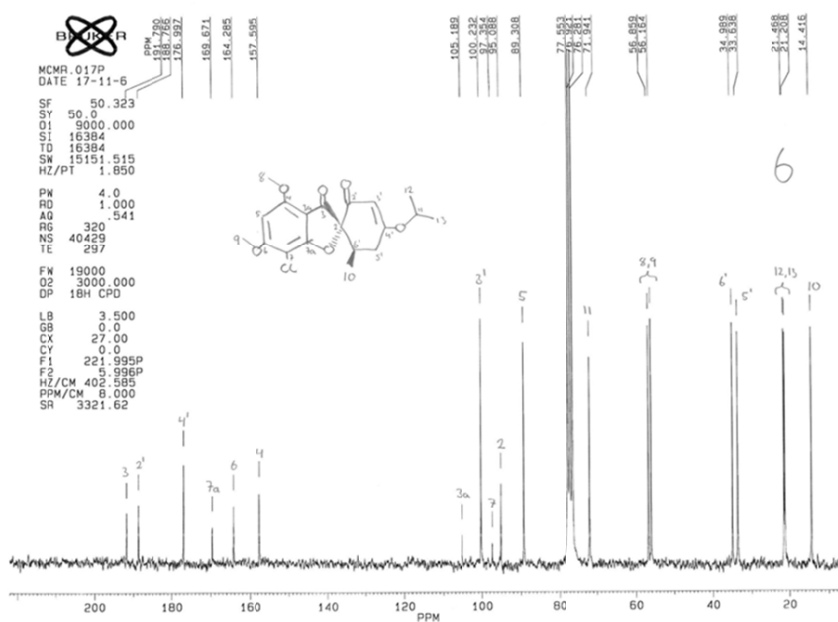
^1H NMR (300 MHz, CDCl_3) **34** ^{13}C NMR (50 MHz, CDCl_3) **34**

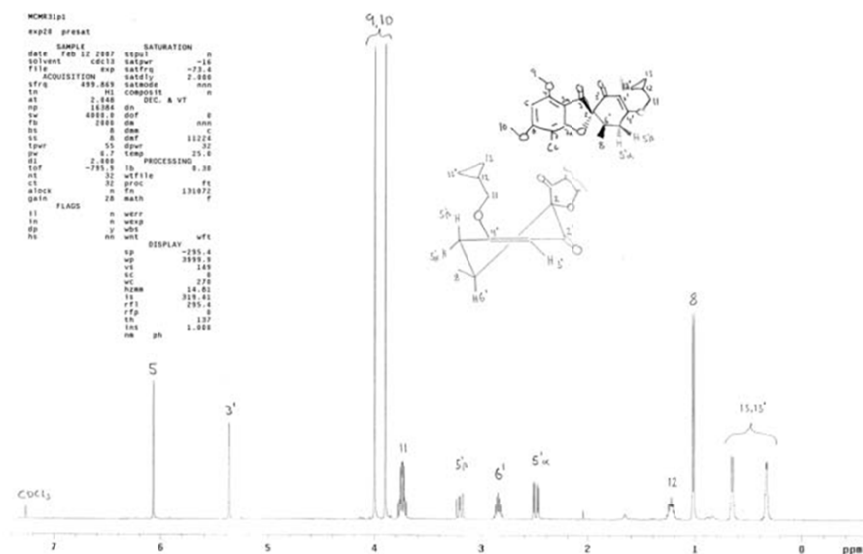
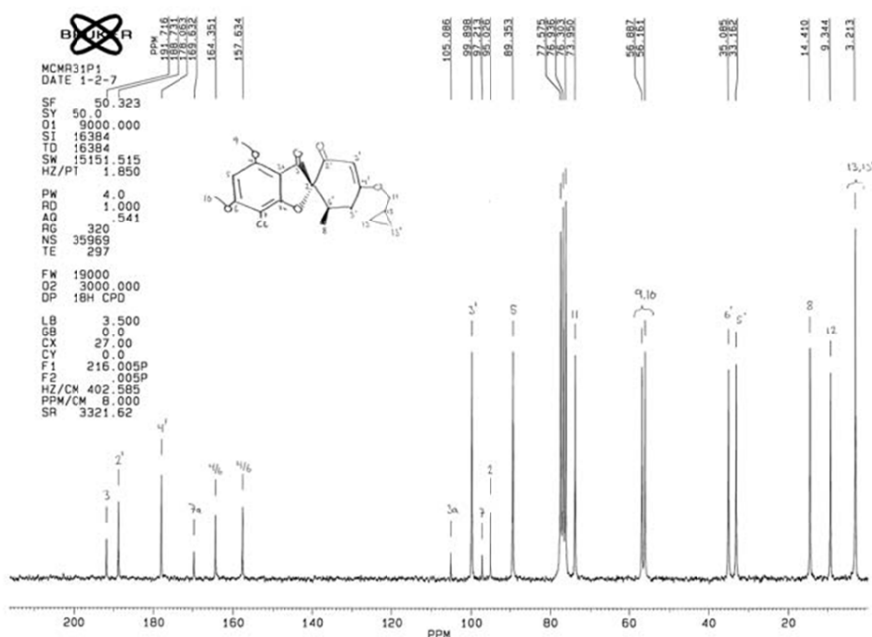
¹H NMR (300 MHz, CDCl₃) **35**¹³C NMR (50 MHz, CDCl₃) **35**

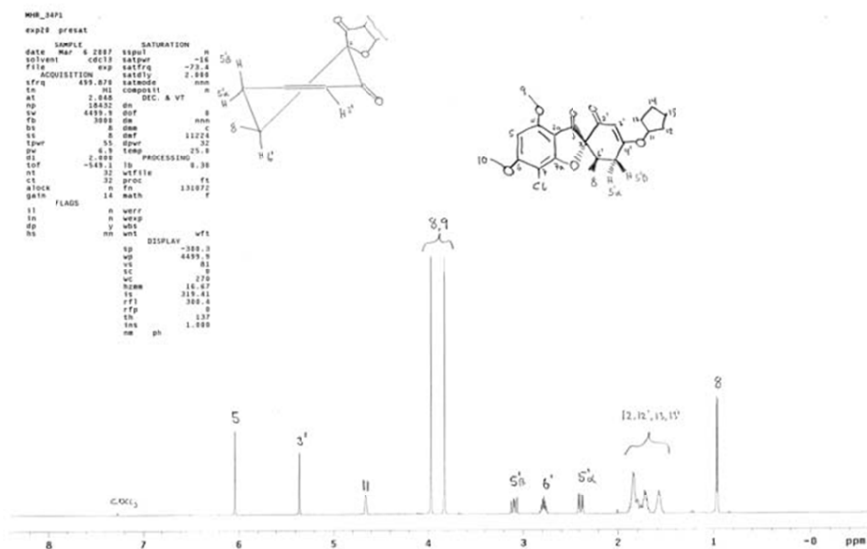
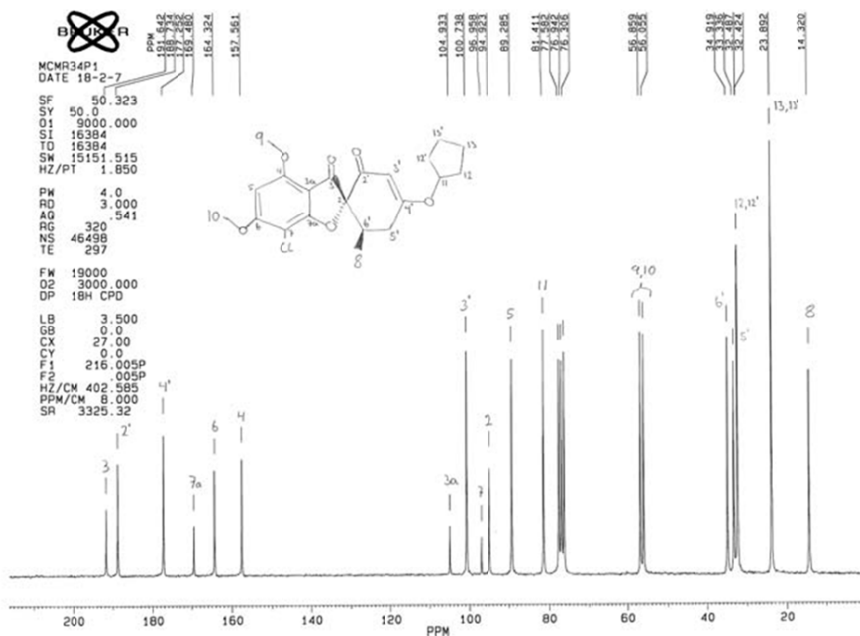
¹H NMR (500 MHz, CDCl₃) **38**¹³C NMR (75 MHz, CDCl₃) **38**

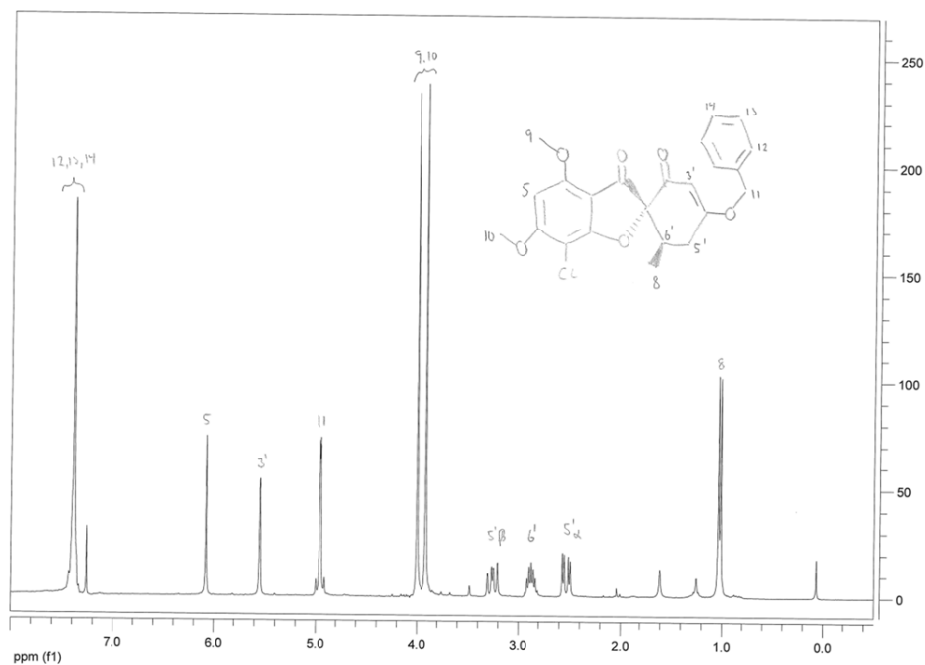
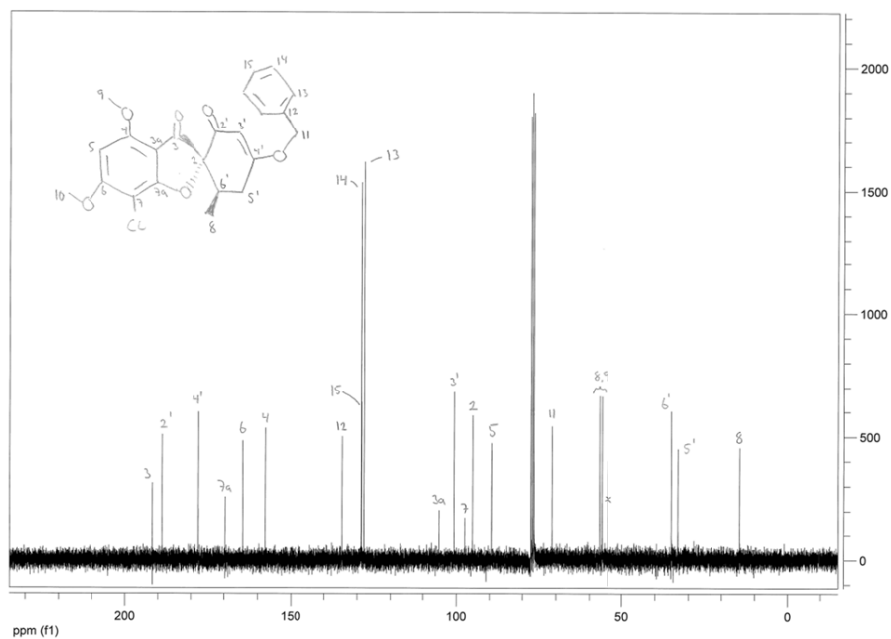
¹H NMR (300 MHz, CDCl₃) **39**¹³C NMR (50 MHz, CDCl₃) **39**

¹H NMR (500 MHz, CDCl₃) **40**¹³C NMR (50 MHz, CDCl₃) **40**

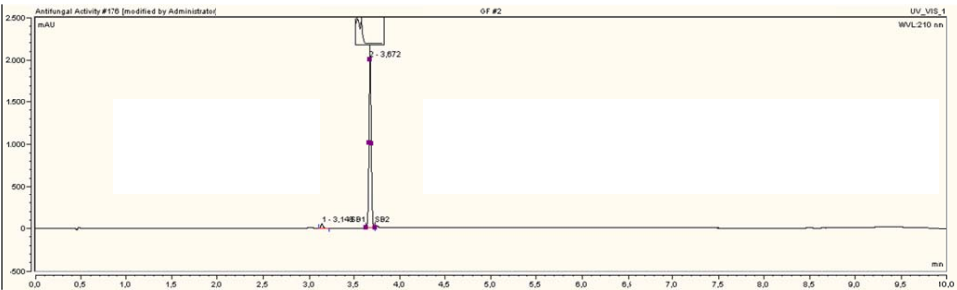
¹H NMR (500 MHz, CDCl₃) **41** ^{13}C NMR (50 MHz, CDCl_3) **41**

¹H NMR (500 MHz, CDCl₃) **42** ^{13}C NMR (50 MHz, CDCl_3) **42**

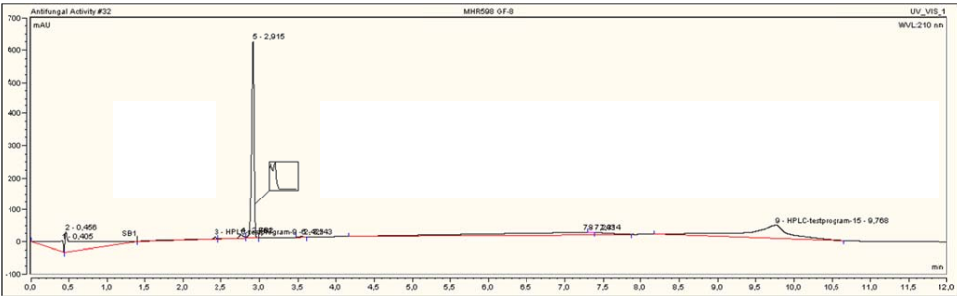
¹H NMR (300 MHz, CDCl₃) **43** ^{13}C NMR (50 MHz, CDCl_3) **43**

¹H NMR (300 MHz, CDCl₃) **44**¹³C NMR (50 MHz, CDCl₃) **44**

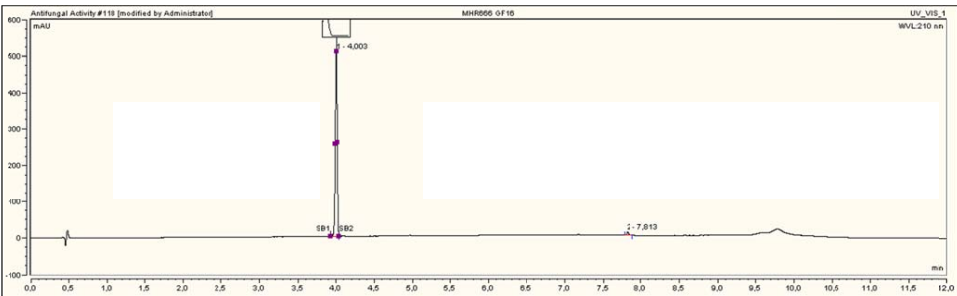
UPLC-DAD Griseofulvin



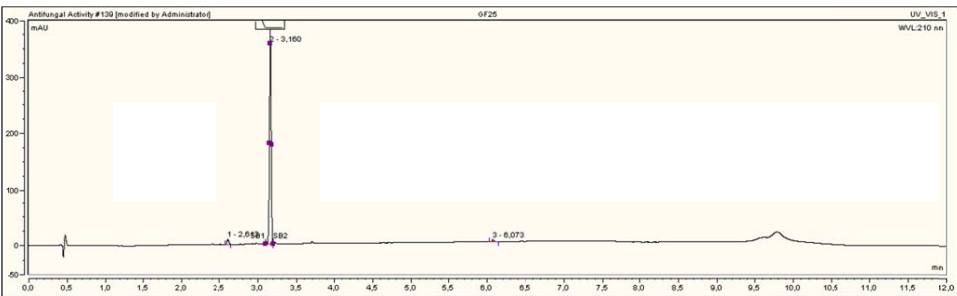
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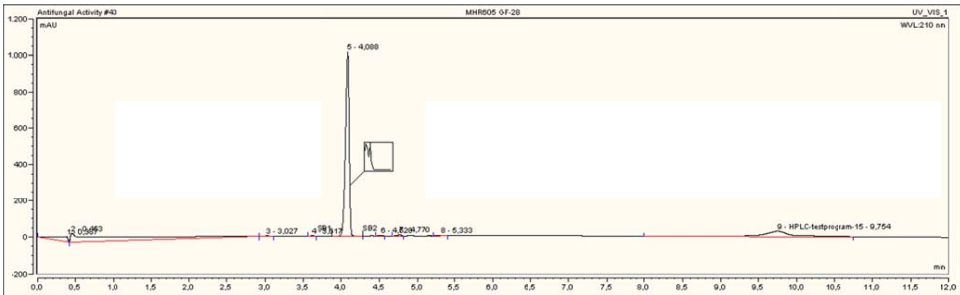
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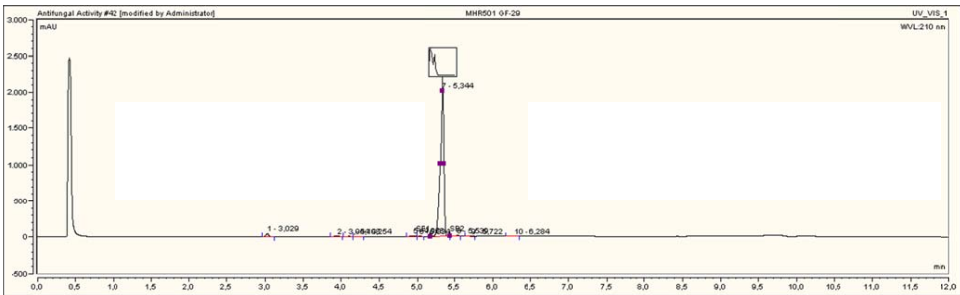
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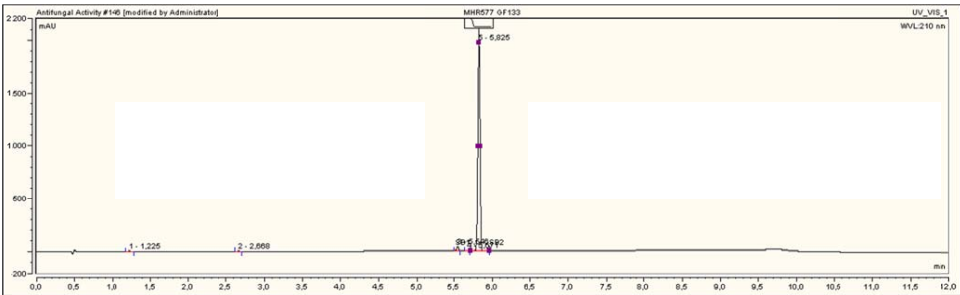
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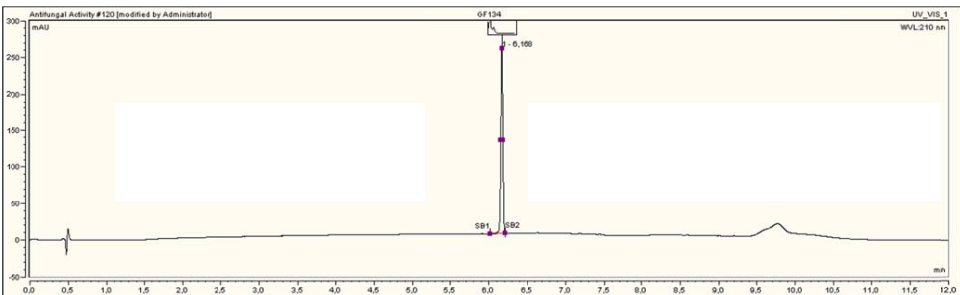
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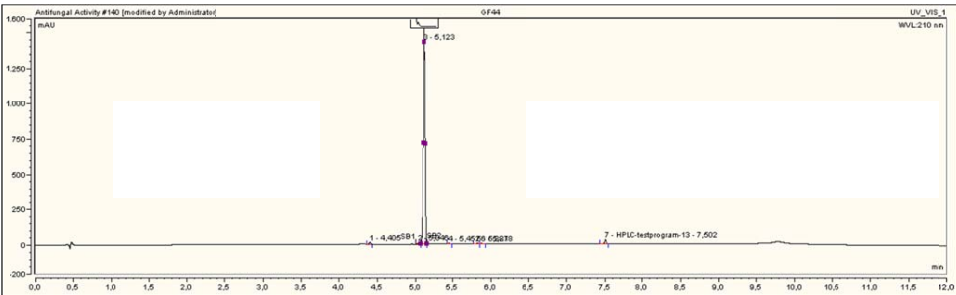
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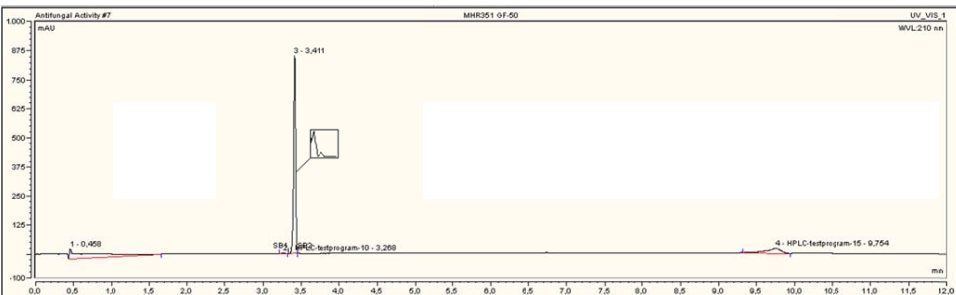
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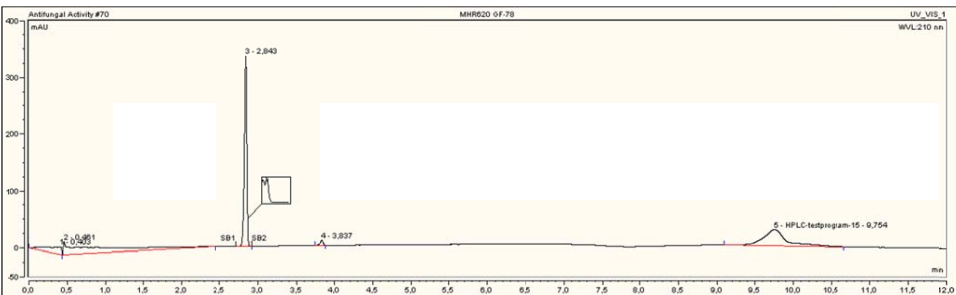
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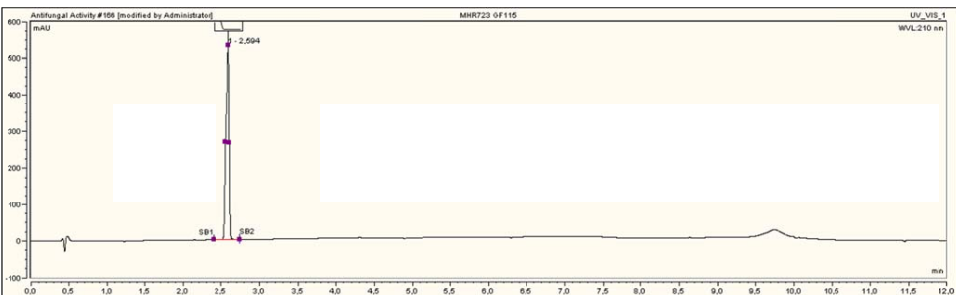
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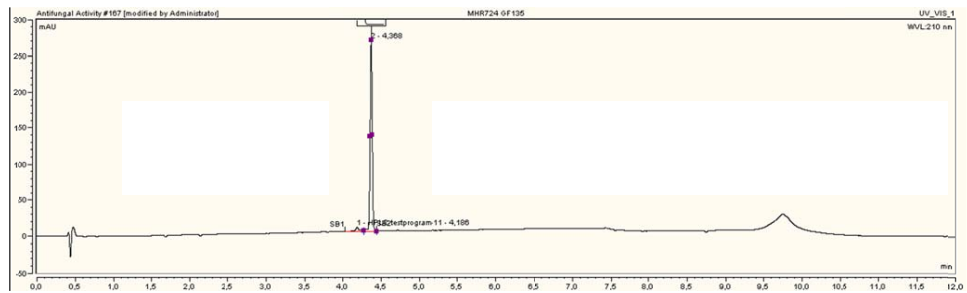
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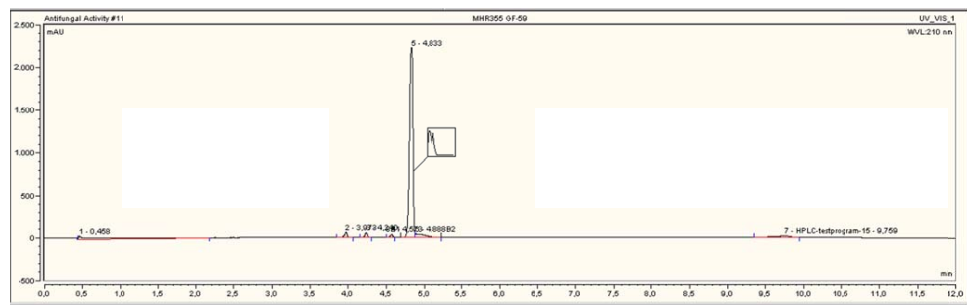
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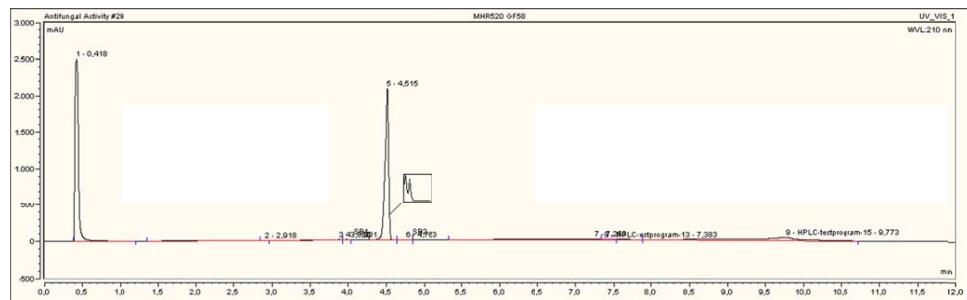
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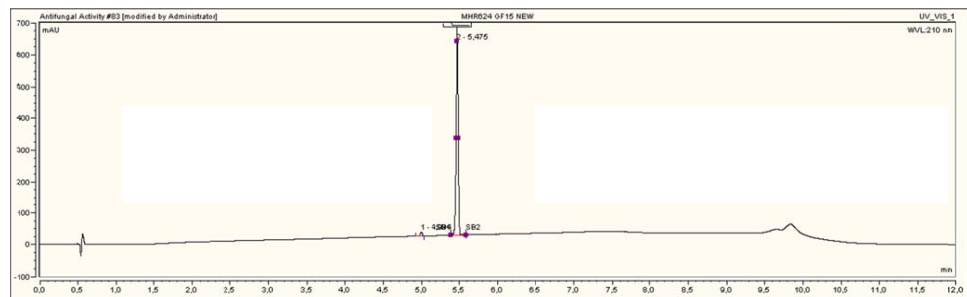
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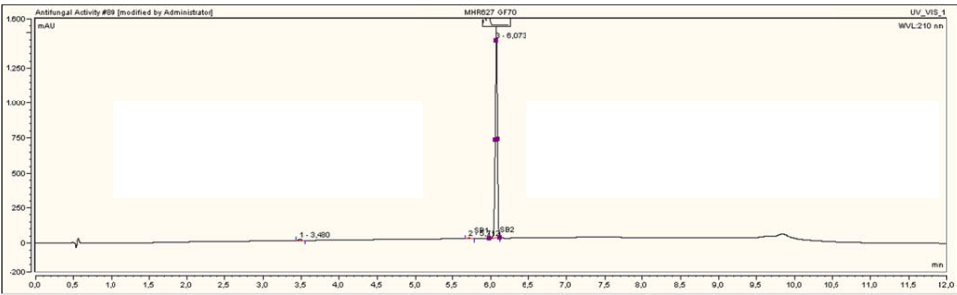
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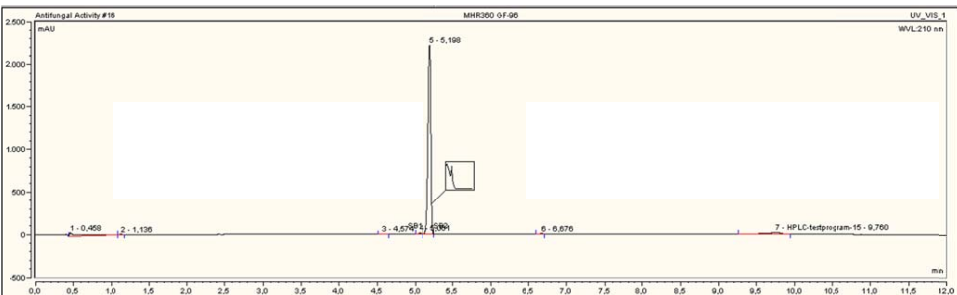
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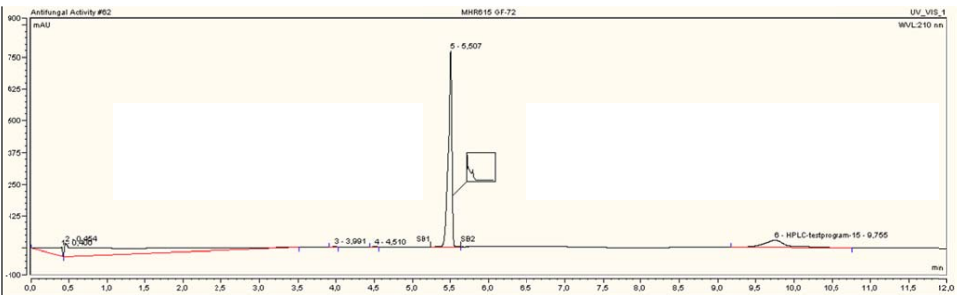
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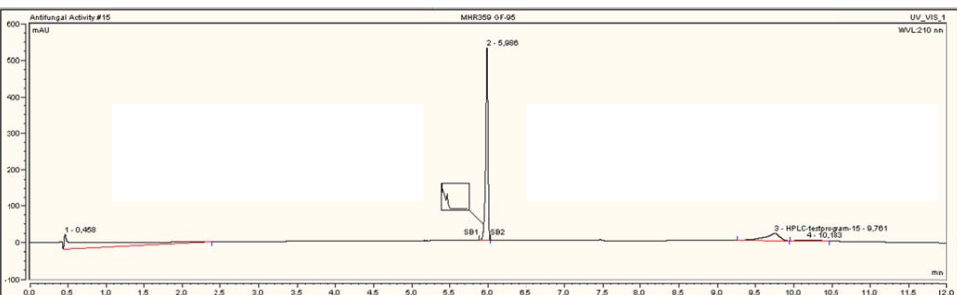
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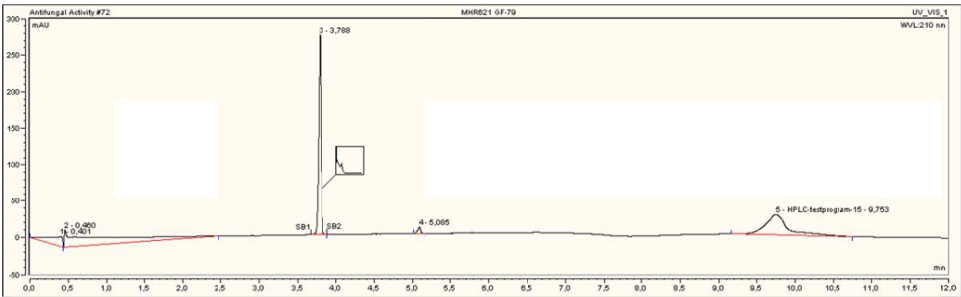
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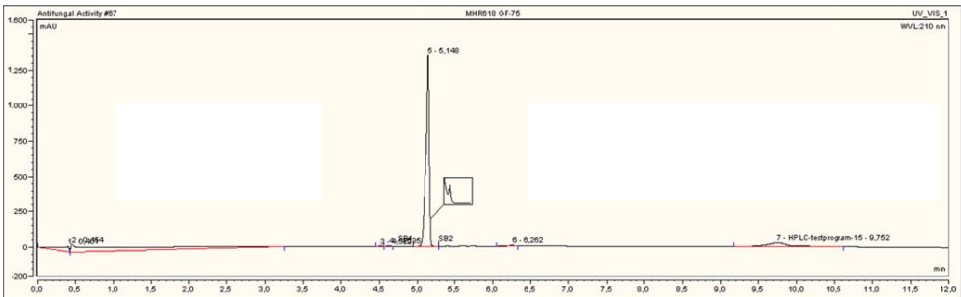
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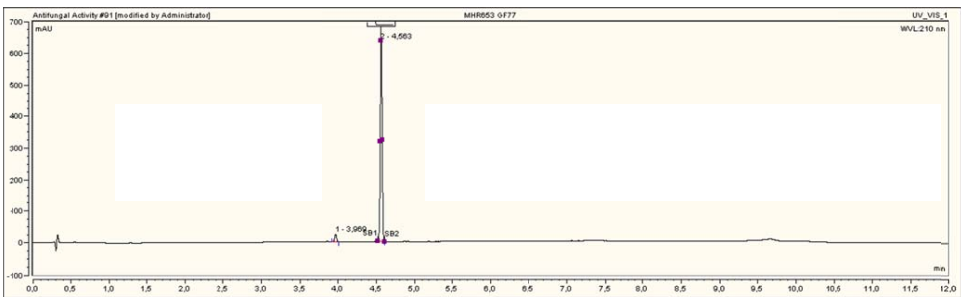
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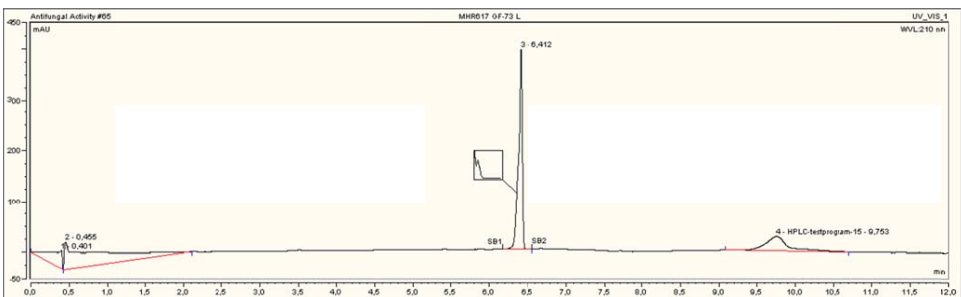
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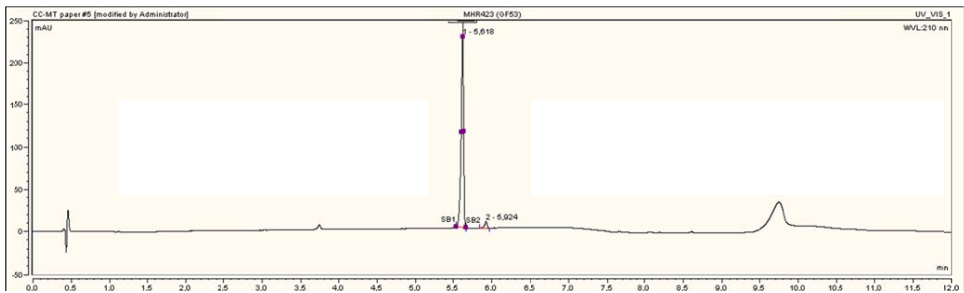
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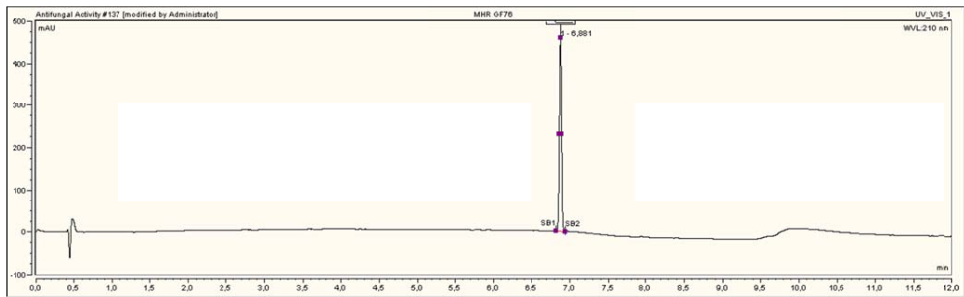
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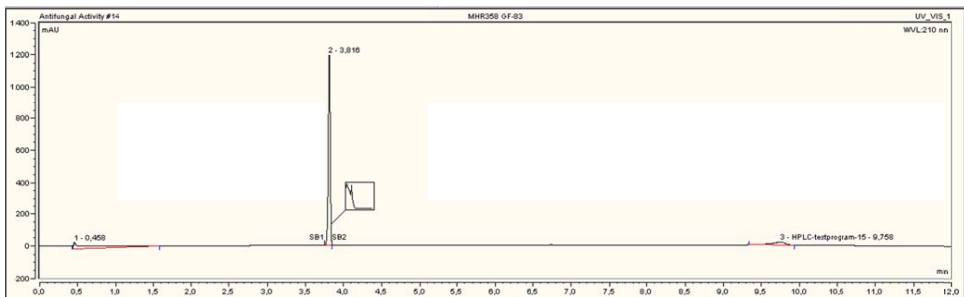
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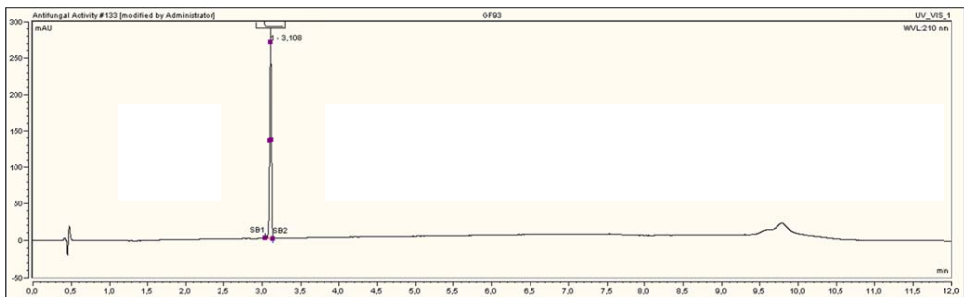
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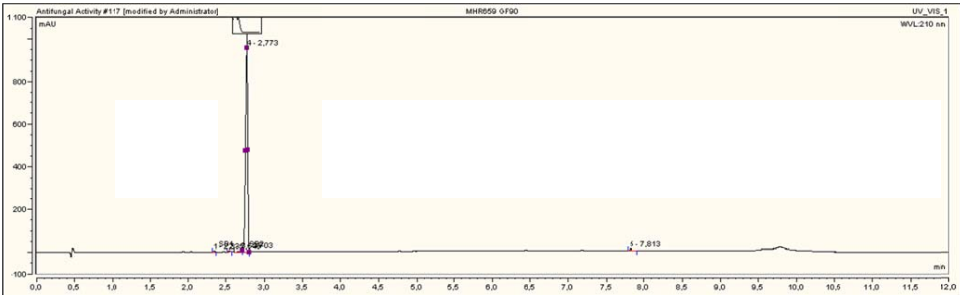
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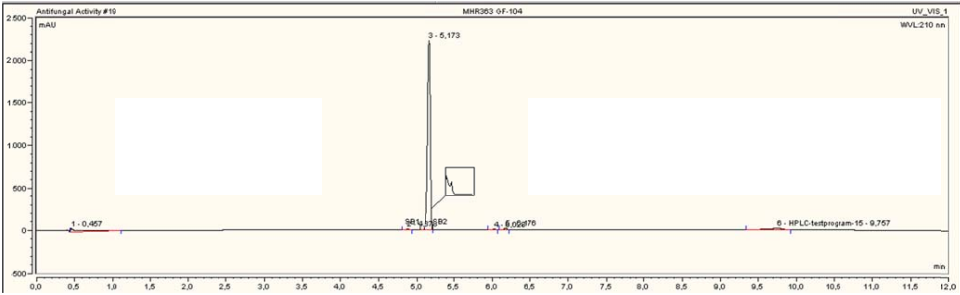
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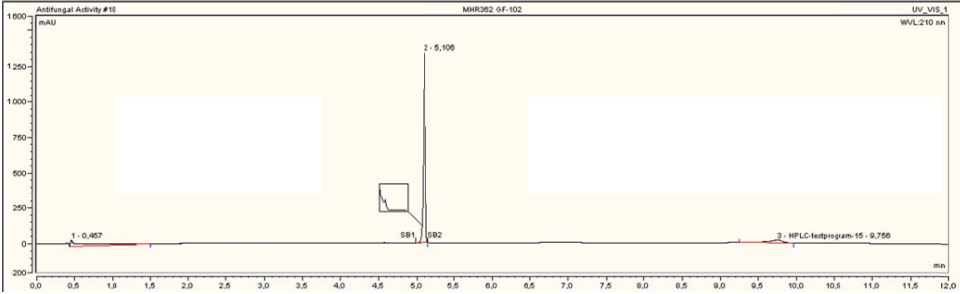
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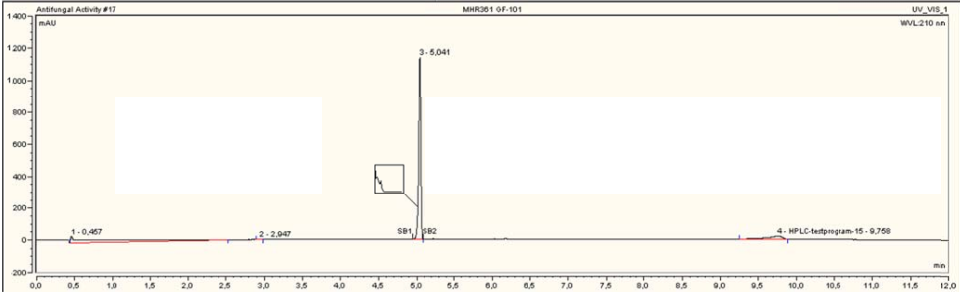
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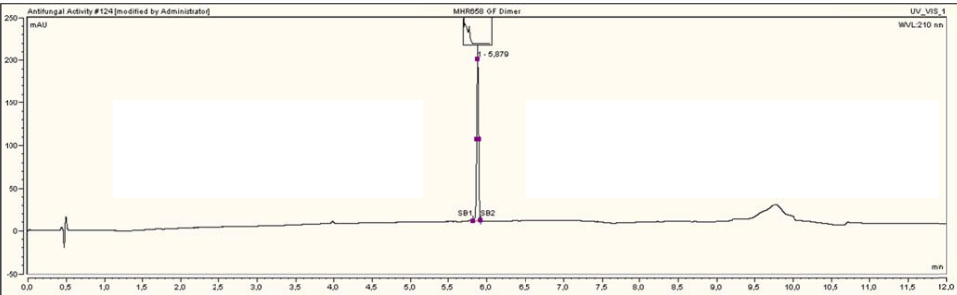
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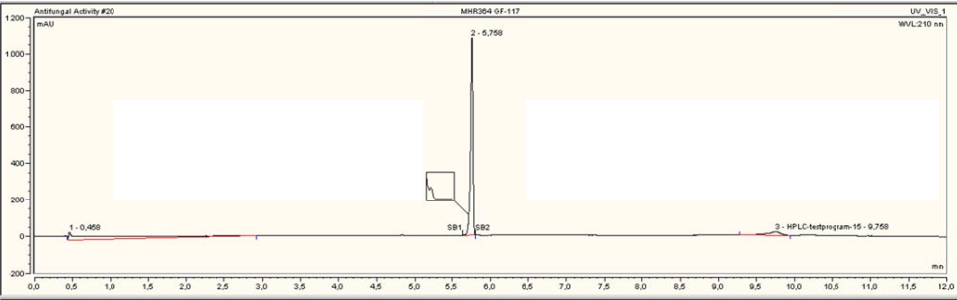
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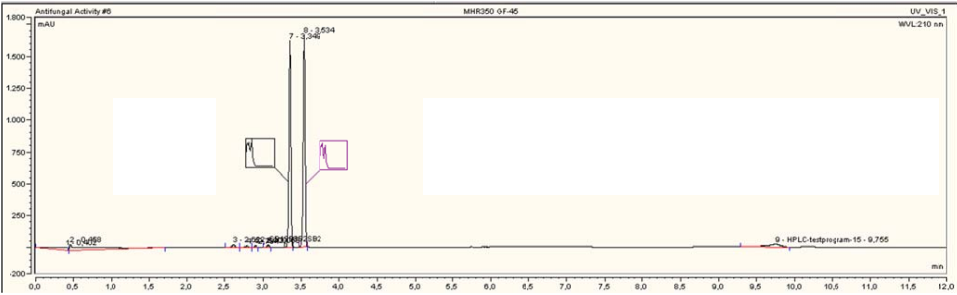
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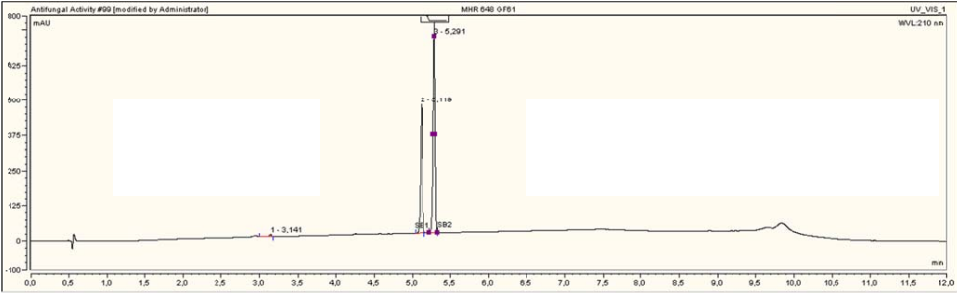
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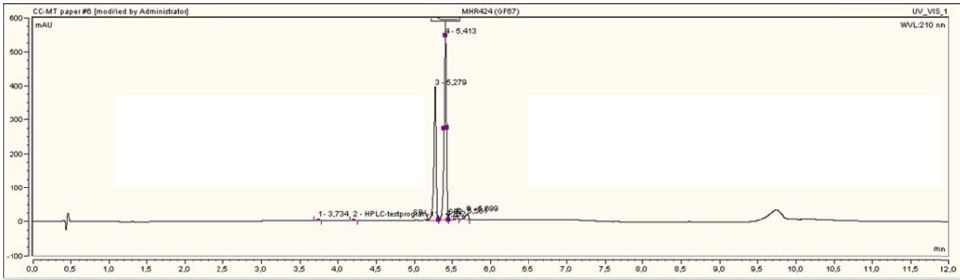
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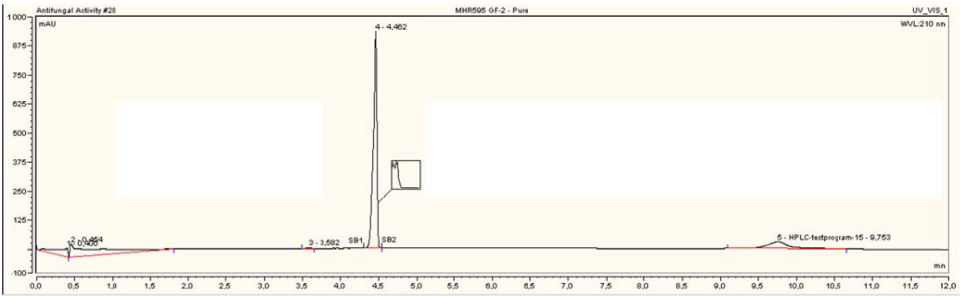
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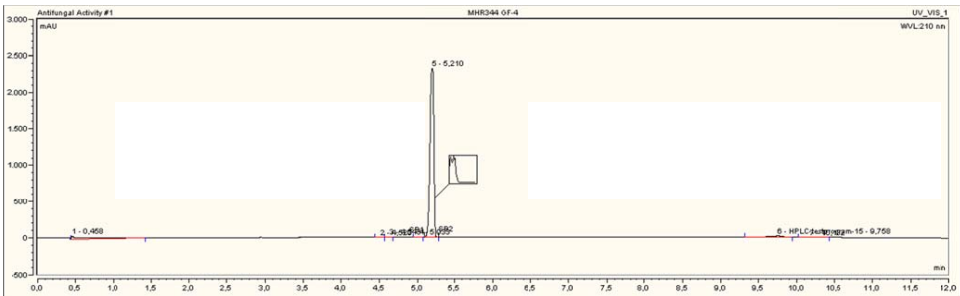
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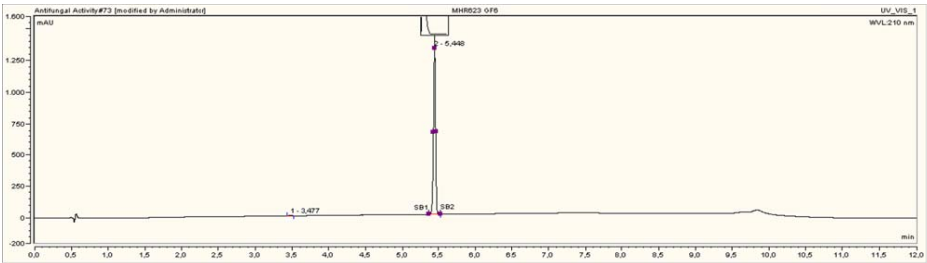
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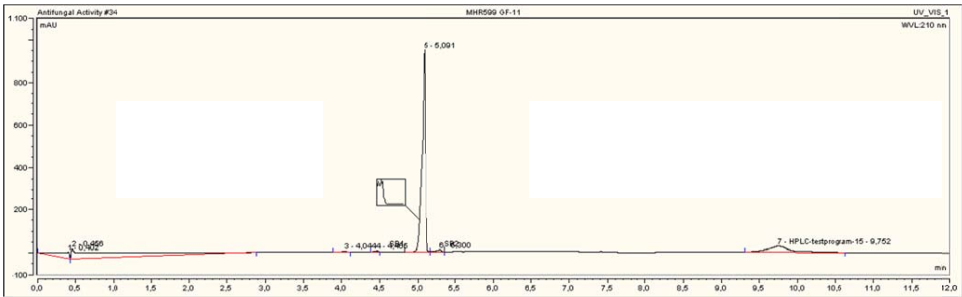
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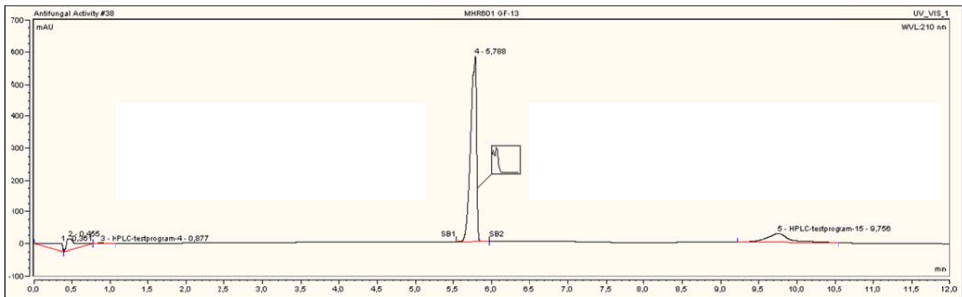
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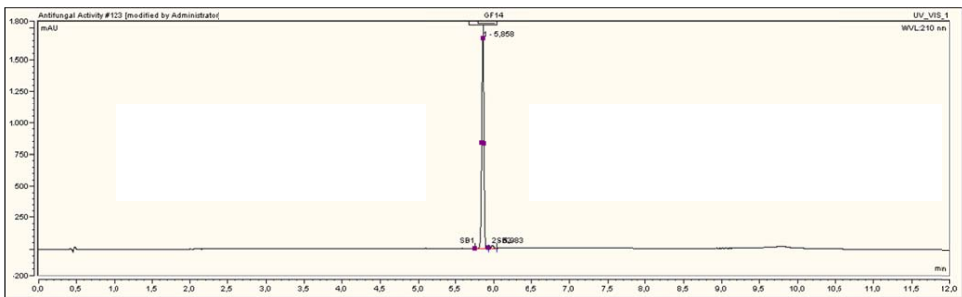
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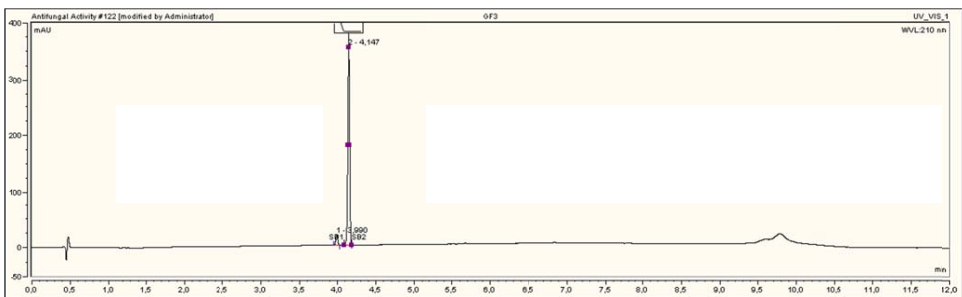
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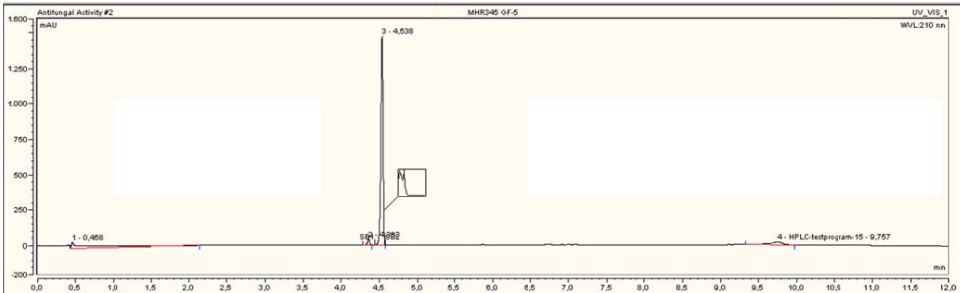
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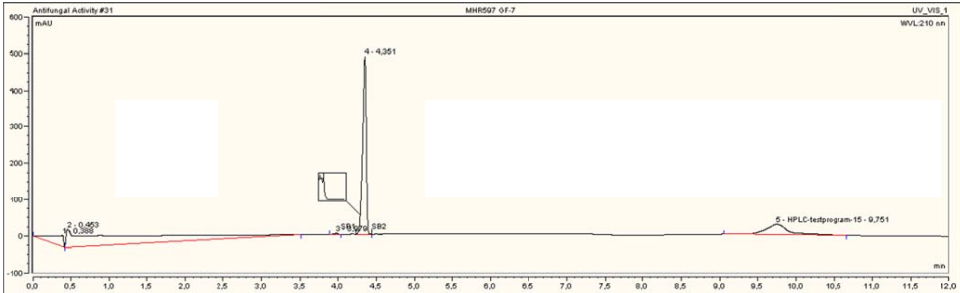
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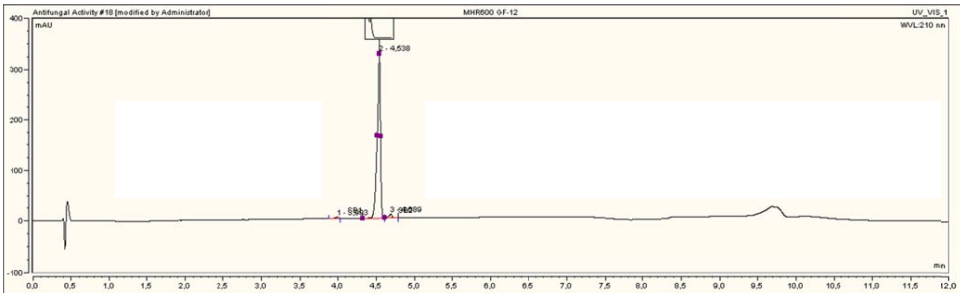
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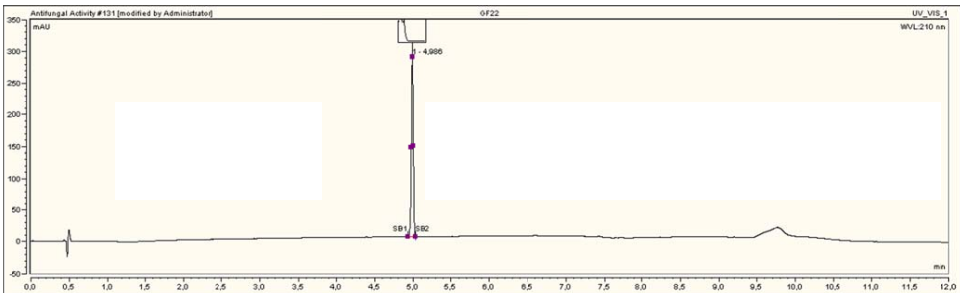
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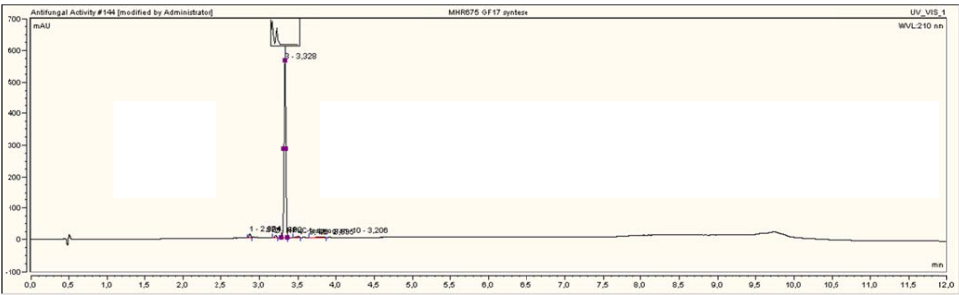
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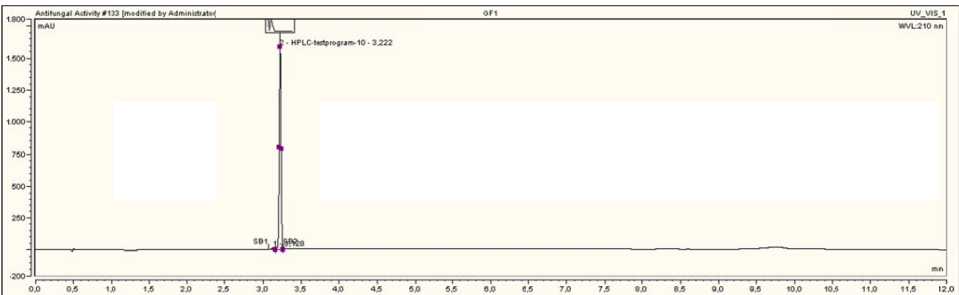
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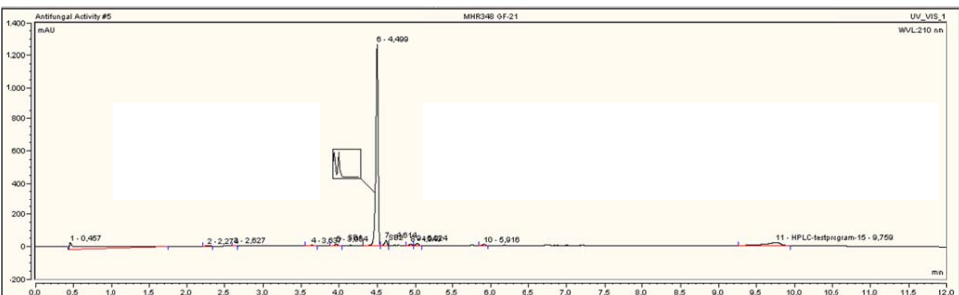
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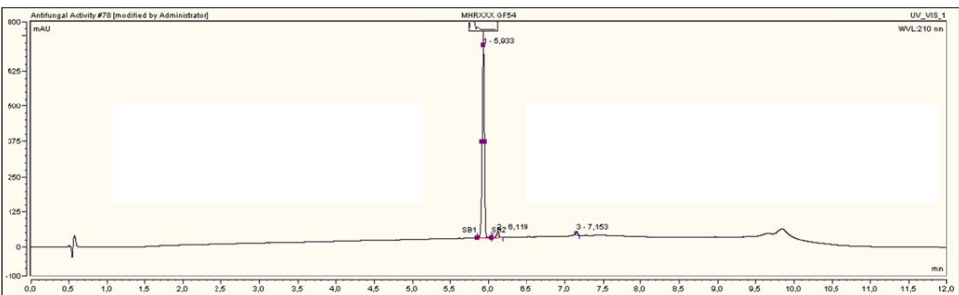
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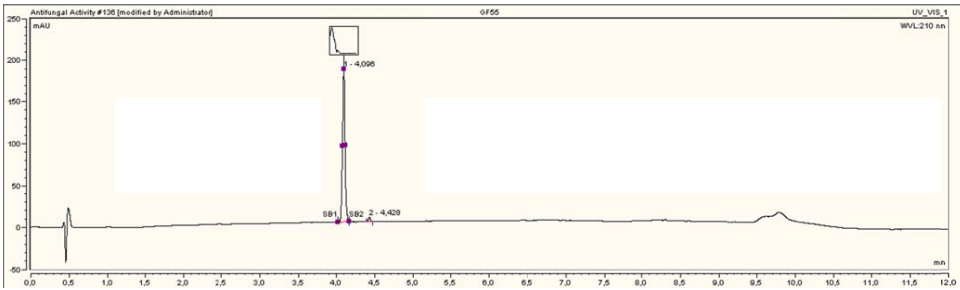
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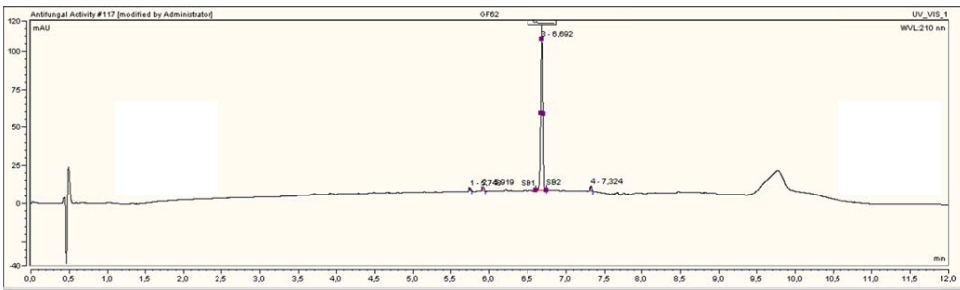
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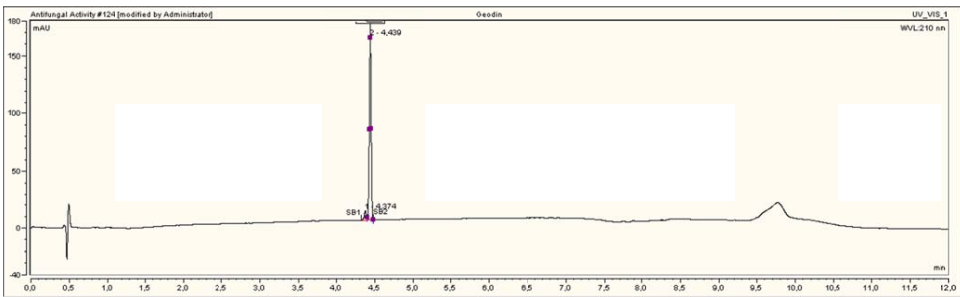
UPLC-DAD 54



UPLC-DAD 55



UPLC-DAD Geodin 56



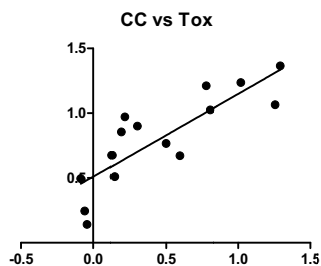


Figure S1 A plot of the IC_{50} (μM) values for compounds showing activity in both the phenotype based assay and the cytotoxicity assay with correlation at $R^2 = 0.70$.

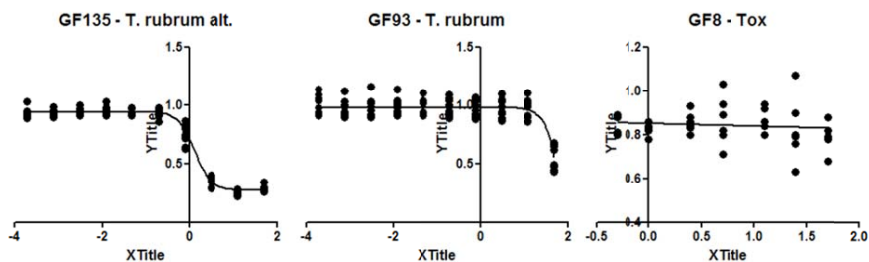


Figure S2. Examples of an active compound (14), a compound with low activity (29) and an inactive compound (2)

References

1. Stephenson, L.; Walker, T.; Warburton, W. K. A.; Webb, G. B. Griseofulvin Analogues. Part IV. The Preparation and Properties of Some Chlorides. *J. Chem. Soc.* **1962**, 1282-1292.
2. Grove, J. F.; Macmillan, J.; Mulholland, T. P. C.; Rogers, M. A. T. Griseofulvin. Part 1. *J. Chem. Soc.* **1952**, 3949-3958.
3. Page, J. E.; Staniforth, S. E. Griseofulvin Analogues. Part V. Infrared Absorption. *J. Chem. Soc.* **1962**, 1292-1303.
4. Duncanson, L. A.; Grove, J. F.; Jeffs, P. W. Griseofulvin. Part XIII. Homologues of Griseofulvin and 7-Chloro-4:6:4'-trimethoxy-6'-methylgris-3'-ene-3:2'-dione. *J. Chem. Soc.* **1958**, 2929-2933.
5. Takeuchi, Y.; Watanabe, I.; Misumi, K.; Irie, M.; Hirose, Y.; Hirata, K.; Yamato, M.; Harayama, T. Syntheses and Antifungal Activity of *dl*-Griseofulvin and Its Congeners. III. *Chem. Pharm. Bull.* **1997**, 45, 2011-2015.

Supporting Information - Chapter 5

Synthesis and single crystal X-ray analysis of two griseofulvin metabolites

Mads H. Rønneest, Pernille Harris, Charlotte H. Gotfredsen, Thomas O. Larsen, Mads H. Clausen*

Supplementary data

S2-S4	Experimental
S5-S10	HPLC chromatograms and 1D NMR spectra of 2 , 3 and S1
S11-S13	gHMBC spectra of 1 , 2 and 3 and 2D assignment of 2 and 3
S14	UV spectra of 2 and 3
S15	Fluorescence data of 2 and 3
S16-S18	MS-MS spectra of 2 and 3 at the collision voltages 30, 45 and 60 V

Experimental

^1H NMR spectra were recorded using either a Varian Unity Inova 500 MHz spectrometer or a Varian Mercury 300 MHz spectrometer. ^{13}C NMR spectra were recorded using a Bruker AC 200 MHz. Chemical shifts were measured in ppm and coupling constants in Hz. When CDCl_3 was used as solvent the residual peak was used as internal reference at δ 7.27 for ^1H NMR and δ 77.00 for ^{13}C NMR spectra. When $\text{DMSO}-d_6$ was used the values were δ 2.50 for ^1H NMR and δ 39.43 for ^{13}C NMR spectra.

IR spectra were recorded using a Bruker Alpha ATR and measured in cm^{-1} . All melting points are uncorrected. TLC was performed on aluminium sheets precoated with silica gel 60 F254 (Merck 1.05554.0001).

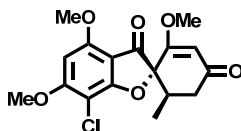
High-resolution LC-DAD-MS was performed on an Agilent 1100 system equipped with a photodiode array detector (DAD) and coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass, Manchester, UK) with a Z-spray electrospray ionisation (ESI) source and a LockSpray probe ($\text{M}+\text{H}$ 556.2771) and controlled by MassLynx 4.0 software. LC-MS calibration from m/z 100-900 was done with a PEG mixture. Standard separation involved a LUNA 2 column with an acetonitrile (50 ppm formic acid) in water gradient starting from 15% to 100% over 25 minutes with a flow rate of 0.3 mL/min.

Compounds were visualized by charring after dipping in a solution of 1% KMnO_4 , 6.7% K_2CO_3 and 0.08% NaOH in water. UV visualization was done using a Model UVGL-25 Mineralight Lamp.

LC-MS/MS analysis was performed with an Agilent HP 1100 liquid chromatography system (Waldbronn, Germany) coupled to a Quattro Ultima triple mass spectrometer (Micromass, Manchester, UK) with ESI source. A Gemini C6-phenyl HPLC column (50×2 mm, $3 \mu\text{m}$) was used with a flow of 0.3 mL/min and $\text{H}_2\text{O}/\text{CH}_3\text{CN}$ (Starting at 80/20, increasing to 0/100 over 9 minutes) as the mobile phase. Tandem mass spectrometry was performed in ESI^+ at a source flow at 700 L/h nitrogen at 350°C . Nitrogen was also used as collision gas, and the MS operated in multiple reaction monitoring mode at the following transitions for both **2** and **3**: daughter ion scan of m/z 339, cone 50 V, collision 30. 45 and 60 V, dwell time 50 ms.

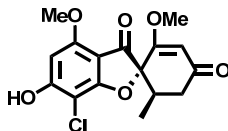
Fluorescence spectra was obtained on an Agilent Series 110 FLD

(2*S*,6'*R*)-(7-Chloro-4,6-dimethoxy-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-en-4'-one) **1**



^1H NMR ($\text{DMSO}-d_6$, 300 MHz) 6.50 (1H, s), 5.60 (1H, s), 4.05 (3H, s), 3.94 (3H, s), 3.62 (3H, s), 2.80 (1H, ddq, $J = 13.3, 6.6, 4.8$ Hz), 2.67 (1H, dd, $J = 16.6, 13.3$ Hz), 2.35 (1H, dd, $J = 16.6, 4.8$ Hz), 0.80 (3H, d, $J = 6.6$ Hz); ^{13}C NMR ($\text{DMSO}-d_6$, 50 MHz) δ 195.5, 191.1, 170.2, 168.5, 164.4, 157.6, 104.6, 104.0, 95.2, 91.3, 90.1, 57.5, 57.0, 56.5, 39.2, 35.5, 13.8.

(2*S*,6'*R*)-(7-Chloro-6-hydroxy-4-methoxy-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methylcyclohex-2'-en-4'-one) **2**



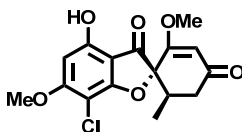
Method A:

Griseofulvin (10 mg, 0.03 mmol) and LiI (4.7 mg, 0.04) was dissolved in pyridine (0.5 mL) and heated to 115 °C. After 16 h the reaction was allowed to reach 20 °C and sat. aq. NH₄Cl was added. The mixture was extracted 3 times with EtOAc, dried (MgSO₄) and concentrated. Purification was performed on a Luna HPLC column (250 × 10 mm, 5 μm, C-18) using 5 mL/min H₂O/CH₃CN (isocratic run at 65/35, for 15 minutes) as the mobile phase to yield **2** (2.8 mg, 29 %) as a yellow oil. *R*_f (CH₂Cl₂:MeOH:HOAc 96:3:1): 0.32; IR(neat): 1707, 1667, 1610; ¹H NMR (DMSO-*d*₆, 500 MHz): 6.26 (1H, s), 5.59 (1H, s), 3.81 (3H, s), 3.63 (3H, s), 2.78 (1H, ddq, *J* = 13.4, 6.5, 4.6 Hz), 2.68 (1H, dd, *J* = 16.4, 13.4 Hz), 2.33 (1H, dd, *J* = 16.4, 4.6 Hz), 0.80 (3H, d, *J* = 6.5 Hz); ¹H NMR(CDCl₃, 500 MHz): δ 6.25 (1H, s), 5.57 (1H, s), 3.92 (3H, s), 3.63 (3H, s), 3.04 (1H, dd, *J* = 16.8, 13.4 Hz), 2.83 (1H, ddq, *J* = 13.4, 6.5, 4.7 Hz), 2.45 (1H, dd, *J* = 16.8, 4.7 Hz), 0.97 (3H, d, *J* = 6.5 Hz); ¹³C NMR(DMSO-*d*₆, from gHSQC and gHMBC, 500 MHz): δ 195.6, 190.6, 170.4, 169.8, 164.5, 156.9, 104.1, 102.9, 94.3, 93.7, 89.8, 56.7, 55.6, 39.2, 35.3, 13.5; ¹³C NMR(CDCl₃, from gHSQC and gHMBC, 500 MHz): δ 197.3, 191.8, 170.6, 169.2, 161.4, 157.9, 105.1, 104.6, 96.9, 93.1, 91.0, 56.4, 56.2, 39.7, 36.0, 13.9; [α]_D²⁵ +254° (c = 0.1 in MeOH); HRMS (ESI⁺) calcd for M+H [C₁₆H₁₆ClO₆]⁺ 339.0635, found 339.0634.

Method B:

Compound **S1** (51 mg, 0.16 mmol) was dissolved in MeOH (2.5 mL), CSA was added (1 mg, 0.01 mmol) and the mixture was refluxed for 20 h. EtOAc was added and the organic phase was washed with sat. aq. NaHCO₃. The aqueous phase was extracted three times with EtOAc and the combined organic phases were dried (MgSO₄) and concentrated. The product **2** was isolated on a Luna HPLC column (250 × 10 mm, 5 μm, C-18) using 5 mL/min H₂O/CH₃CN (isocratic run at 65/35, for 15 minutes) as the mobile phase to yield **2** (1 mg, 2 %)

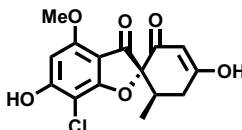
(2*S*,6'*R*)-(7-Chloro-6-methoxy-4-hydroxy-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methylcyclohex-2'-en-4'-one) **3**



A solution of MgI₂ was prepared by adding magnesium turnings (31 mg, 1.3 mmol) and iodine (54 mg, 0.43 mmol) to anhydrous Et₂O (2 mL) and toluene (1 mL). This solution was sonicated at 60 °C for 90 min., filtered and added to griseofulvin (**1**) (100 mg, 0.29 mmol) and heated to 80 °C. The solution was cooled to 20 °C after 20 h. and 0.2 M H₂SO₄ was added. The mixture was extracted 3 times with EtOAc, dried (MgSO₄) and concentrated. The residue was purified by column chromatography (CH₂Cl₂:MeOH:AcOH 97:2:1), yielding **3**, which was recrystallized from EtOAc/heptane to afford yellow crystals (95 mg, 98%). *R*_f (CH₂Cl₂:MeOH:HOAc 96:3:1): 0.25; m.p: 138-140 °C; IR(neat): 1697, 1620, 1449; ¹H NMR(DMSO-*d*₆, 500 MHz): δ 11.53 (1H, s), 6.26 (1H, s), 5.60 (1H, s), 3.92 (3H, s), 3.63 (3H, s), 2.78 (1H, ddq, *J* = 13.4, 6.5, 4.5 Hz), 2.68 (1H, dd, *J* = 16.3, 13.4 Hz), 2.34 (1H, dd, *J* = 16.3, 4.5 Hz), 0.81 (3H, d, *J* = 6.5 Hz); ¹H NMR(CDCl₃, 300 MHz): δ 6.18 (1H, s), 5.57 (1H, s), 3.97 (3H, s), 3.65 (3H, s), 2.95 (1H, dd, *J* = 15.3, 13.1 Hz), 2.92-

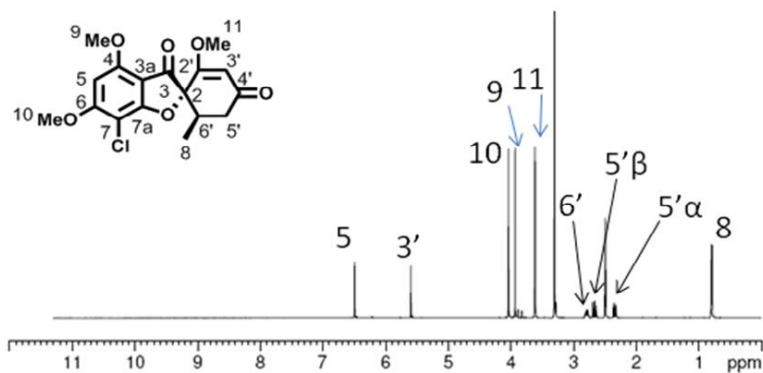
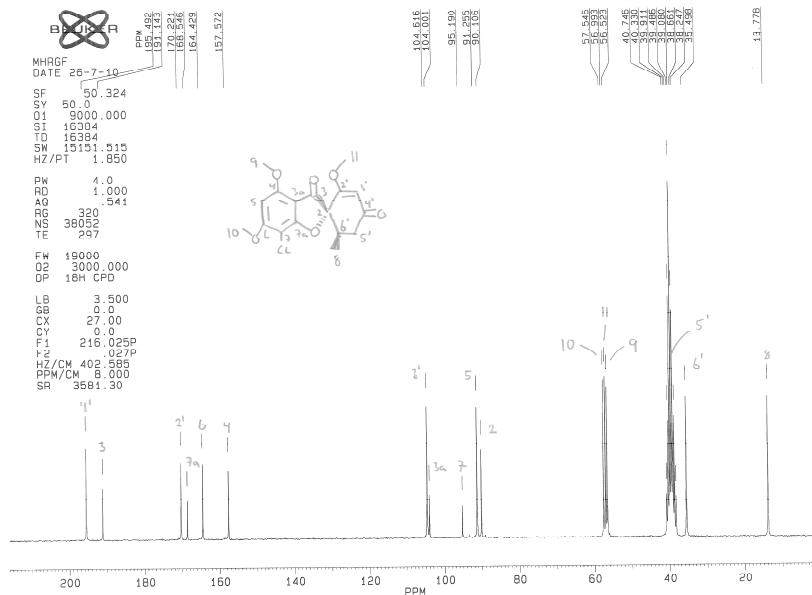
2.80 (1H, m), 2.48 (1H, dd, $J = 15.3, 3.2$ Hz), 0.97 (3H, d, $J = 6.4$ Hz); ^{13}C NMR(DMSO- d_6 , 500 MHz): δ 195.5, 191.3, 170.4, 168.1, 163.7, 156.8, 104.5, 103.7, 94.1, 93.6, 89.8, 56.9 (2C), 39.4, 35.6, 13.8; ^{13}C NMR(CDCl₃, 50 MHz): δ 196.8, 195.6, 170.4, 167.3, 165.5, 156.0, 104.8, 103.9, 96.4, 93.7, 91.2, 57.2, 56.7, 40.0, 36.2, 14.2; $[\alpha] +310^\circ$ ($c = 0.3$ in CCl₃); HRMS (ESI⁺) calcd for M+H [C₁₆H₁₆ClO₆]⁺ 339.0635, found 339.0633.

(2*S*,6'*R*)-(7-Chloro-6-hydroxy-4-methoxy-benzofuran-3-on)-2-spiro-1'-(6'-methyl-cyclohex-2',4'-dione) **S1**



Griseofulvic acid^{1,2} (300 mg, 0.88 mol) was dissolved in 0.66 M NaOH (10 mL) and heated to 100 °C for 24 H. The mixture was allowed to reach 20 °C and filtered. The supernatant was acidified with 2 M H₂SO₄ and the precipitate purified on reverse phase silica (MeOH/H₂O). The compound was re-crystallized from MeOH/H₂O to afford **S1** (123 mg, 43%) as white crystals. R_f (CH₂Cl₂:MeOH:HOAc 89:10:1): 0.49; m.p: 259-261 °C (decomp.); IR(neat): 3100 (b), 1661, 1632, 1579, 1538; ^1H NMR(DMSO- d_6): δ 11.88 (1H, s), 6.24 (1H, s), 5.30 (1H, s), 3.78 (3H, s), 2.88-2.66 (2H, m), 2.52-2.40 (1H, m), 0.84 (3H, d, $J = 6.1$ Hz); ^{13}C NMR(DMSO- d_6): δ 190.7, 187.8, 179.3, 170.1, 163.7, 156.9, 103.5, 101.5, 94.3 (2C), 93.6, 55.9, 34.4, 32.9 14.3; $[\alpha] +289^\circ$ ($c = 0.3$ in MeOH); HRMS (ESI⁺) calcd for M+H [C₁₅H₁₄ClO₆]⁺ 325.0479, found 325.0467.

1. Rønneest, M. H.; Rebacz, B.; Markworth, L.; Terp, A. H.; Larsen, T. O.; Krämer, A.; Clausen, M. H. *J. Med. Chem.* **2009**, 52, 3342.
2. Arkley, V.; Gregory, G. I.; Attenbur, J.; Walker, T. *J. Chem. Soc.* **1962**, 1260.

Griseofulvin **1** ^1H NMR (500 MHz, $\text{DMSO}-d_6$)Figure 1. ^1H NMR of **1** in DMSO ^{13}C NMR (50 MHz, $\text{DMSO}-d_6$)

(2*S*,6'*R*)-(7-Chloro-6-hydroxy-4-methoxy-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-en-4'-one) **2**

^1H NMR (500 MHz, $\text{DMSO}-d_6$)

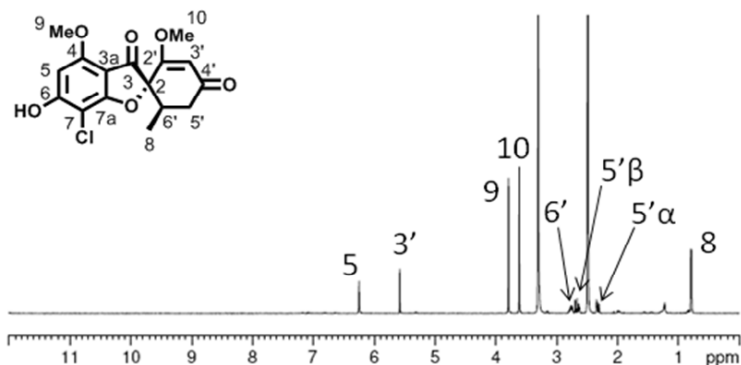
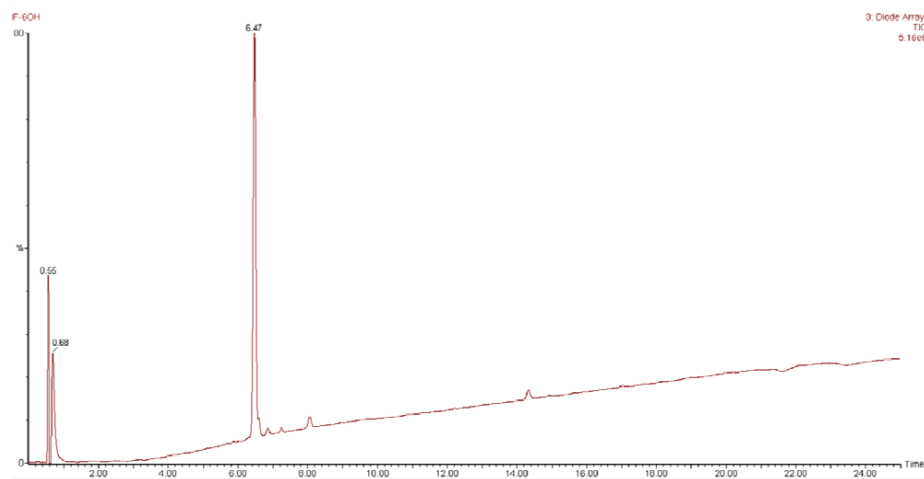


Figure 2. ^1H NMR of **2** in DMSO

LC-DAD chromatogram



(2*S*,6'*R*)-(7-Chloro-6-methoxy-4-hydroxy-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methylcyclohex-2'-en-4'-one) **3**

^1H NMR (500 MHz, $\text{DMSO}-d_6$)

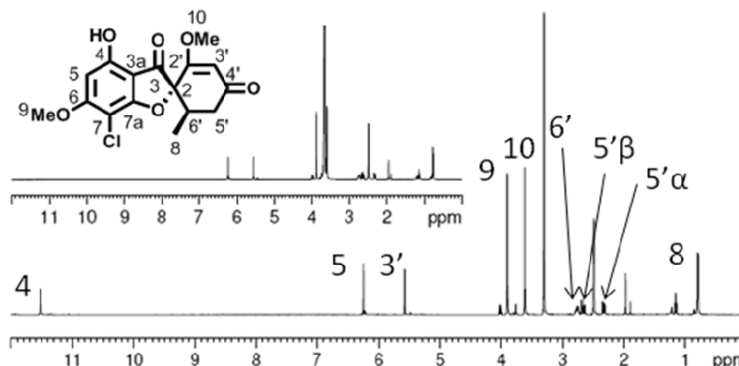
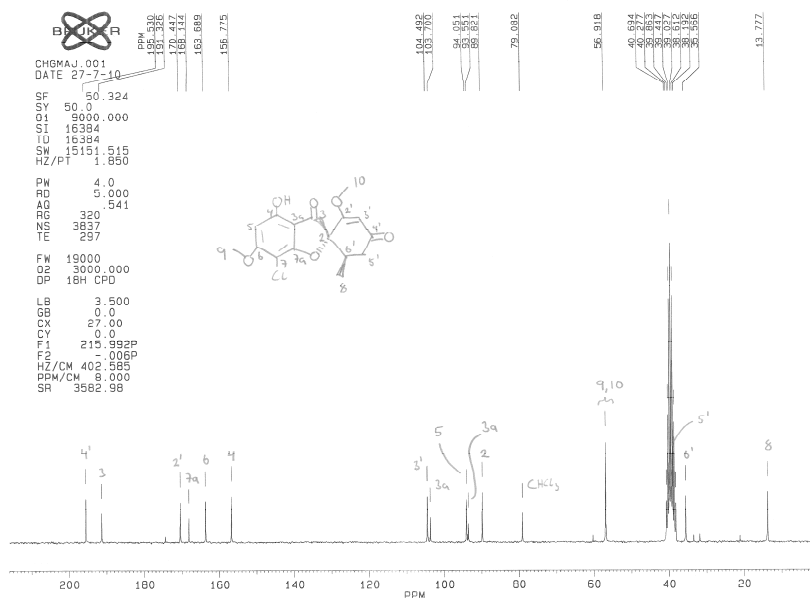
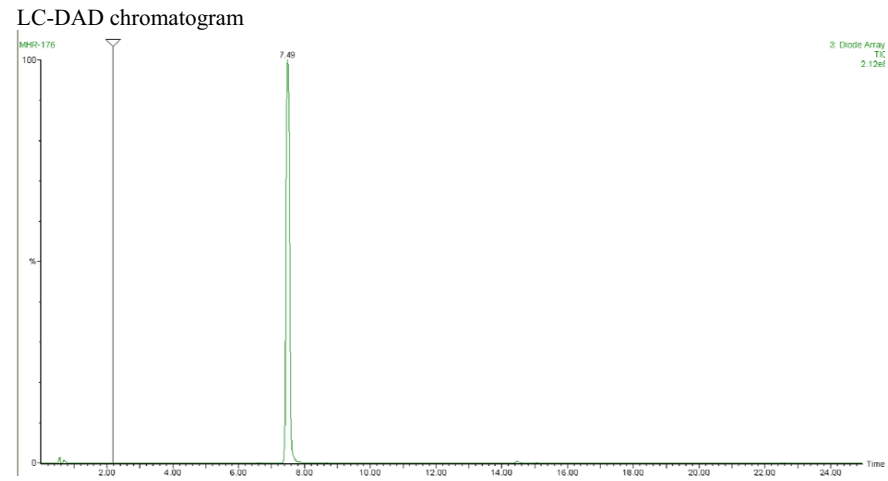


Figure 3. ^1H NMR of **3** in DMSO , insert showing spectrum after shaking with D_2O .

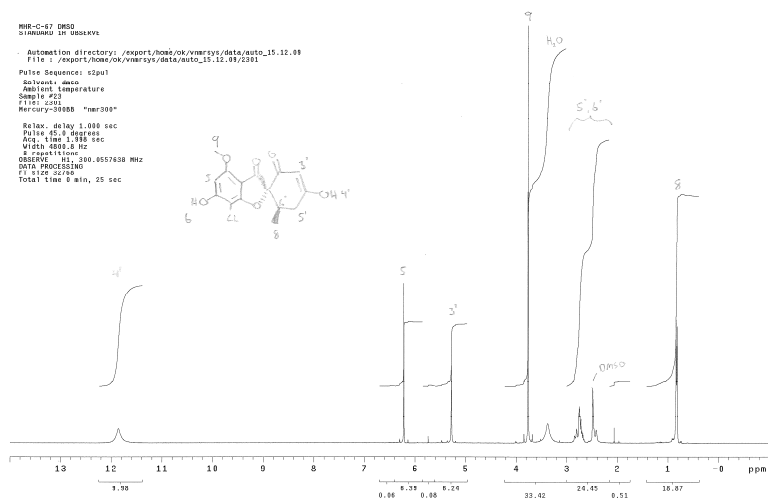
^{13}C NMR (50 MHz, $\text{DMSO}-d_6$)



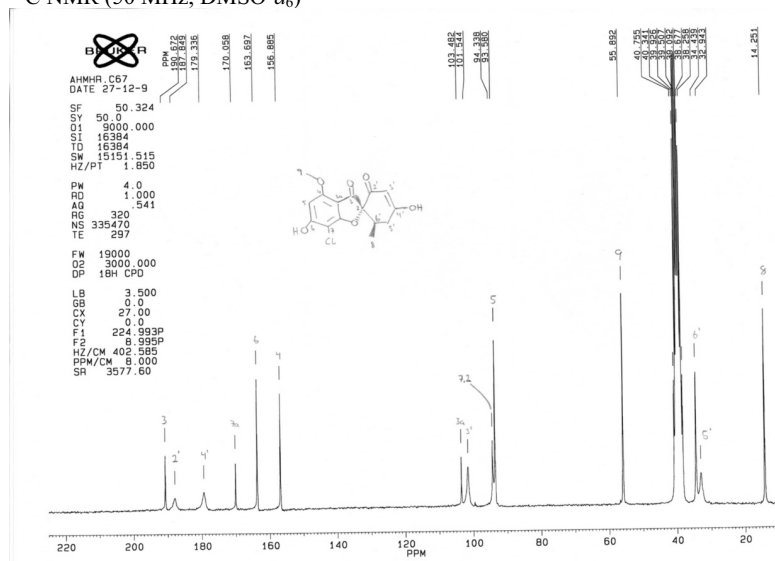


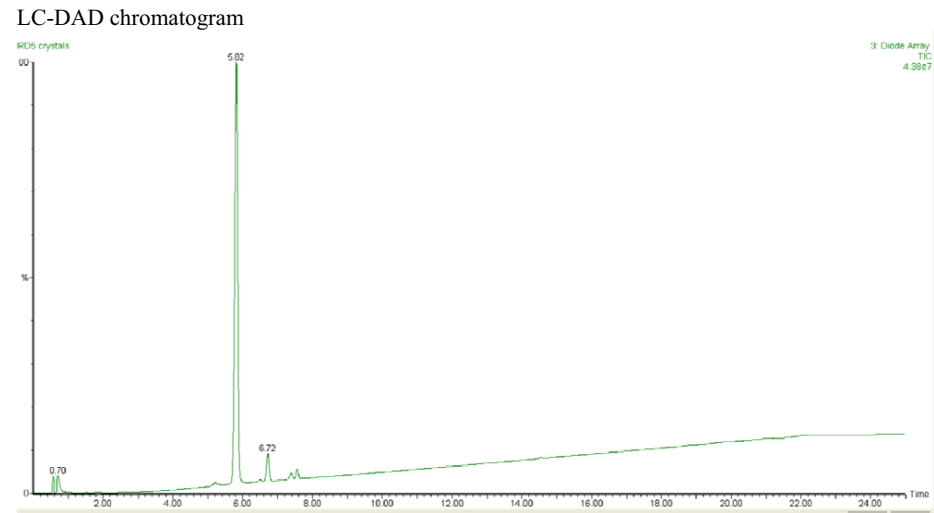
(2*S*,6'*R*)-(7-Chloro-6-hydroxy-4-methoxy-benzofuran-3-on)-2-spiro-1'-(6'-methyl-cyclohex-2',4'-dione) **S1**

^1H NMR (300 MHz, DMSO- d_6)



^{13}C NMR (50 MHz, DMSO- d_6)





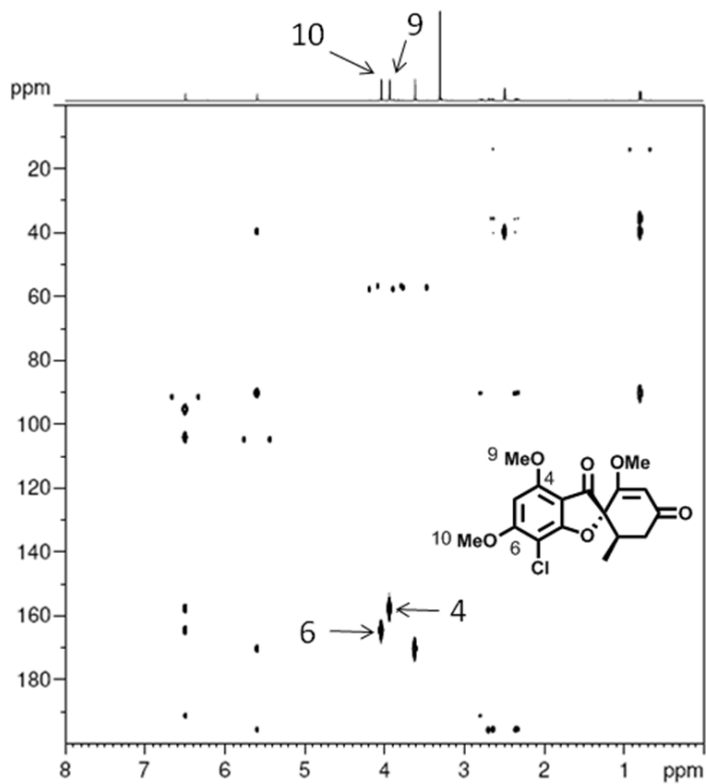
Griseofulvin **1**gHMBC (500 MHz, DMSO-*d*₆)

Figure 4. gHMBC spectrum of griseofulvin, showing that the two methoxy groups (9 and 10) only have one correlation each, which is to the corresponding ipso carbon.

(2*S*,6'*R*)-(7-Chloro-6-hydroxy-4-methoxy-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-en-4'-one) **2**

Atom#	¹ H NMR	¹³ C NMR	gHMBC	DQF-COSY
2		89.8		
3		190.6		
3a		102.9		
4		156.9		
5	6.26 (1H, s)	93.7	3, 3a, 4, 6, 7, 7a	
6		164.5		
7		94.3		
7a		169.8		
8	0.80 (3H, d, <i>J</i> = 6.5 Hz)	13.5	2, 4', 5', 6'	6'
9	3.81 (3H, s)	55.6	4	
10	3.63 (3H, s)	56.7	2', 3'	
2'		170.4		
3'	5.59 (1H, s)	104.1	2, 2', 4', 5'	
4'		195.6		
5'α	2.33 (1H, dd, <i>J</i> = 16.4, 4.6 Hz)	39.2	2, 3', 4', 6', 8	5'β, 6'
5'β	2.68 (1H, dd, <i>J</i> = 16.4, 13.4 Hz)		2, 4', 6', 8	5'α, 6'
6'	2.78 (1H, ddq, <i>J</i> = 13.4, 6.5 4.6 Hz)	35.3	2, 3, 5', 8	5'α, 5'β, 8

Table 1. ¹H NMR as well as gHMBC and DQF-COSY data for **2** (500 MHz, DMSO-*d*₆)

(2*S*,6'*R*)-(7-Chloro-6-methoxy-4-hydroxy-benzofuran-3-on)-2-spiro-1'-(2'-methoxy-6'-methyl-cyclohex-2'-en-4'-one) **3**

gHMBC (500 MHz, DMSO-*d*₆)

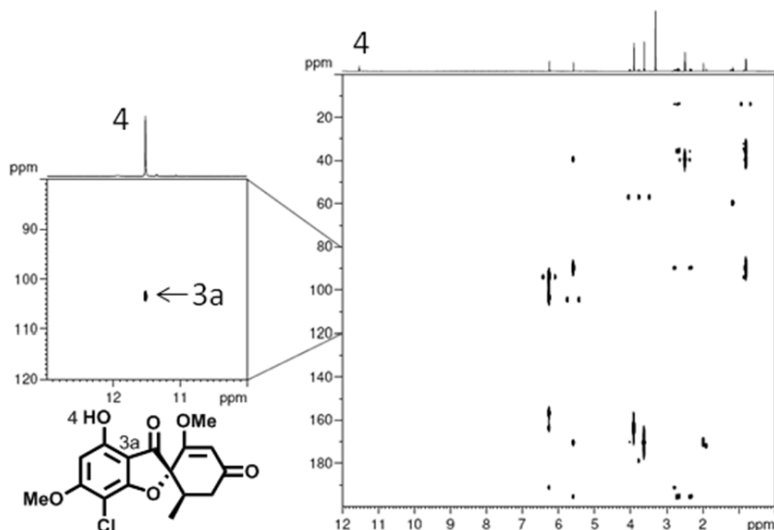
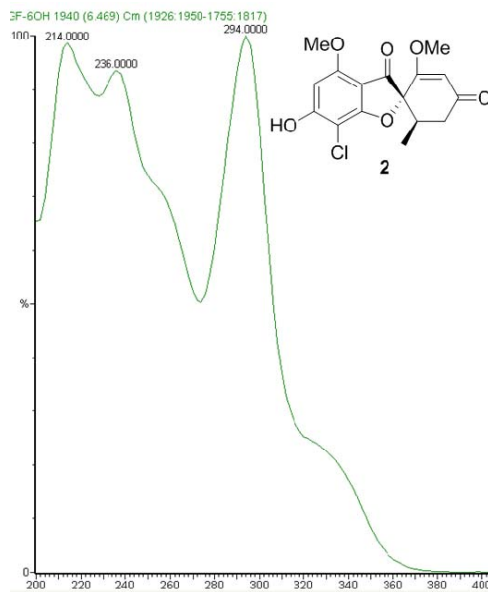
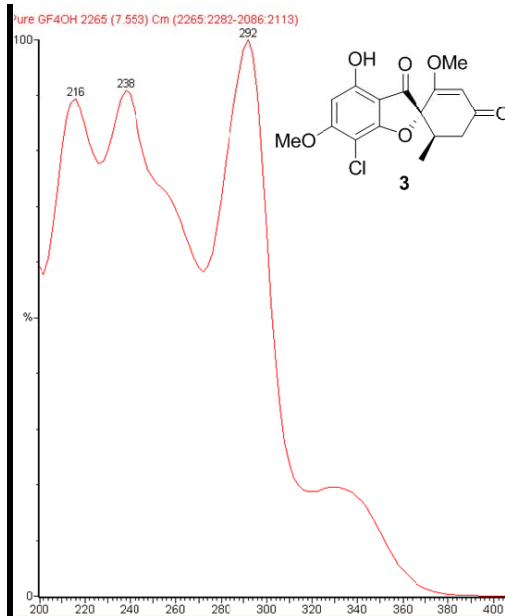


Figure 5. gHMBC of **3**. By increasing the intensity, a correlation can be seen from the phenol (**4**) to the carbon shift 103.7, which corresponds to the **3a** position, indicating the position of the phenol.

Atom#	¹ H NMR	¹³ C NMR	gHMBC	DQF-COSY
2		89.8		
3		191.3		
3a		103.7		
4	11.53 (1H, s)	156.8	3a	
5	6.26 (1H, s)	94.1	3, 3a, 4, 6, 7, 7a	
6		163.7		
7		93.6		
7a		168.1		
8	0.81 (3H, d, <i>J</i> = 6.5 Hz)	13.8	2, 4', 5', 6'	6'
9	3.92 (3H, s)	56.9	6	
10	3.63 (3H, s)	56.9	2', 3'	
2'		170.4		
3'	5.60 (1H, s)	104.5	2, 2', 4', 5'	
4'		195.5		
5'α	2.34 (1H, dd, <i>J</i> = 16.3, 4.5 Hz)	39.4	2, 3', 4', 6', 8	5'β, 6'
5'β	2.68 (1H, dd, <i>J</i> = 16.3, 13.4 Hz)		2, 4', 6', 8	5'α, 6'
6'	2.78 (1H, ddq, <i>J</i> = 13.4, 6.5 4.5 Hz)	35.6	2, 2', 3, 5', 8	5'α, 5'β, 8

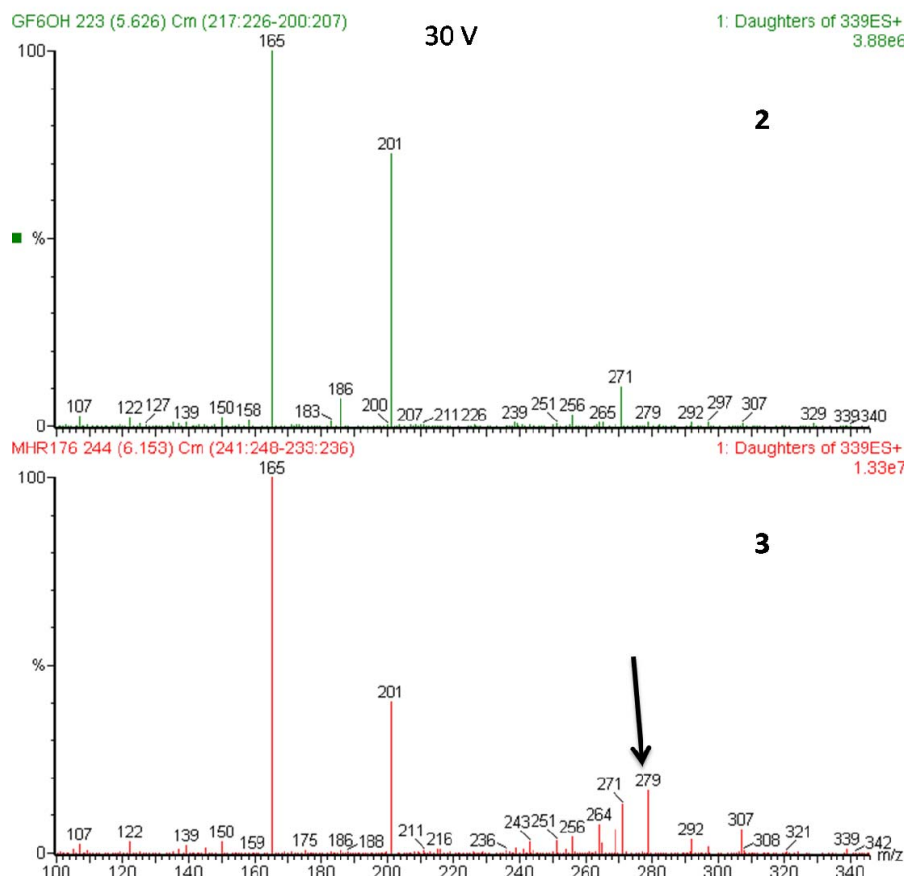
Table 2. ¹H and ¹³C NMR as well as gHMBC and DQF-COSY data **3** (500 MHz, DMSO-*d*₆)

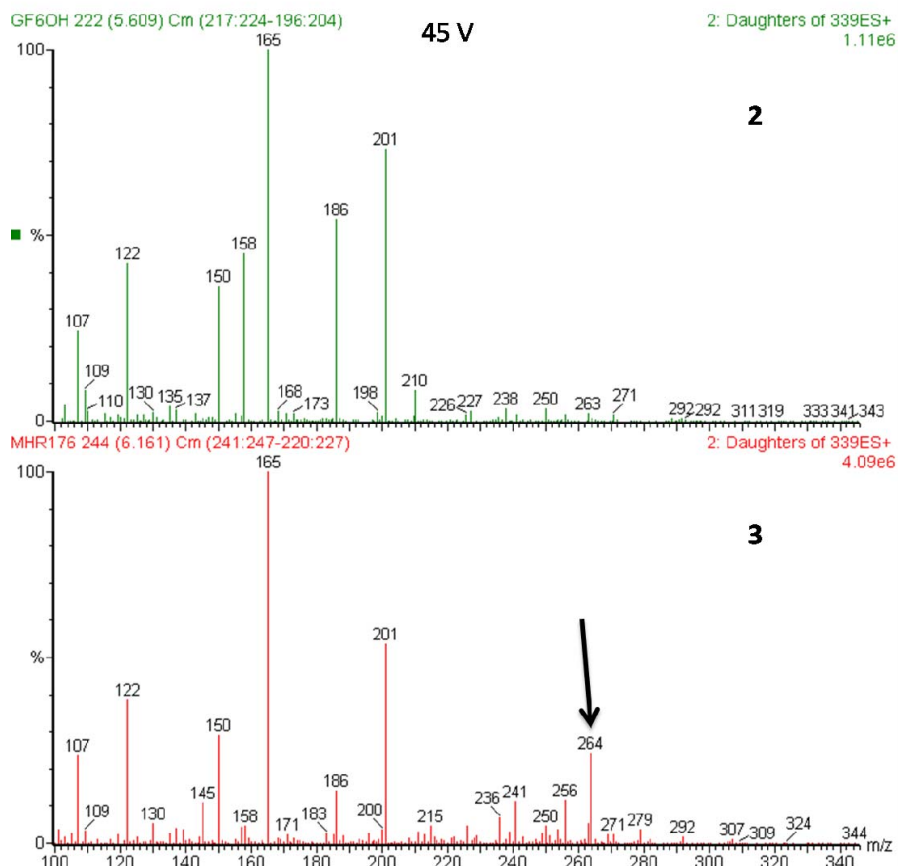
UV spectrum of **2**UV spectrum of **3**

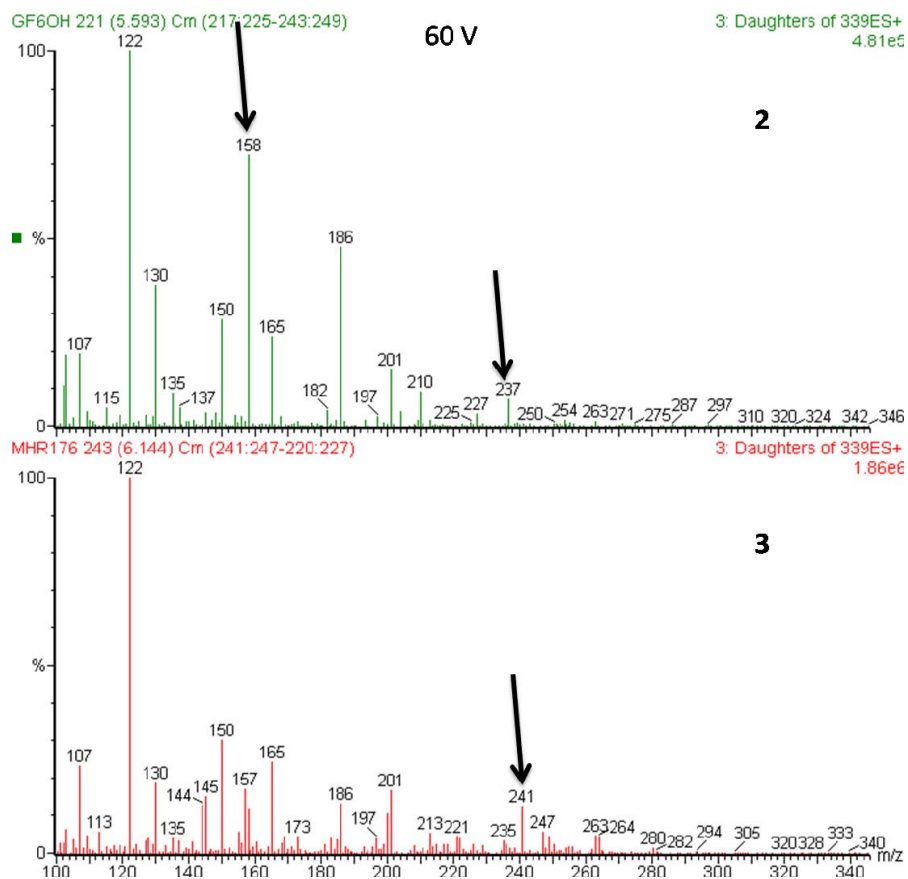
Fluorescence data

Excitation	Emission (2)	Emission (3)
215 nm	420 nm	424 nm
237 nm	424 nm	430 nm
295 nm	427 nm	434 nm

MS-MS spectra of **2** and **3** (collision 30 V)



MS-MS spectra of **2** and **3** (collision 45 V)

MS-MS spectra of **2** and **3** (collision 60 V)

Supporting Information - Chapter 6

A technique for selective *ipso*-substitution of *ortho*-, *meta*- or *para*-aryl trimethylsilyl groups with radioactive iodide in quantitative yields.

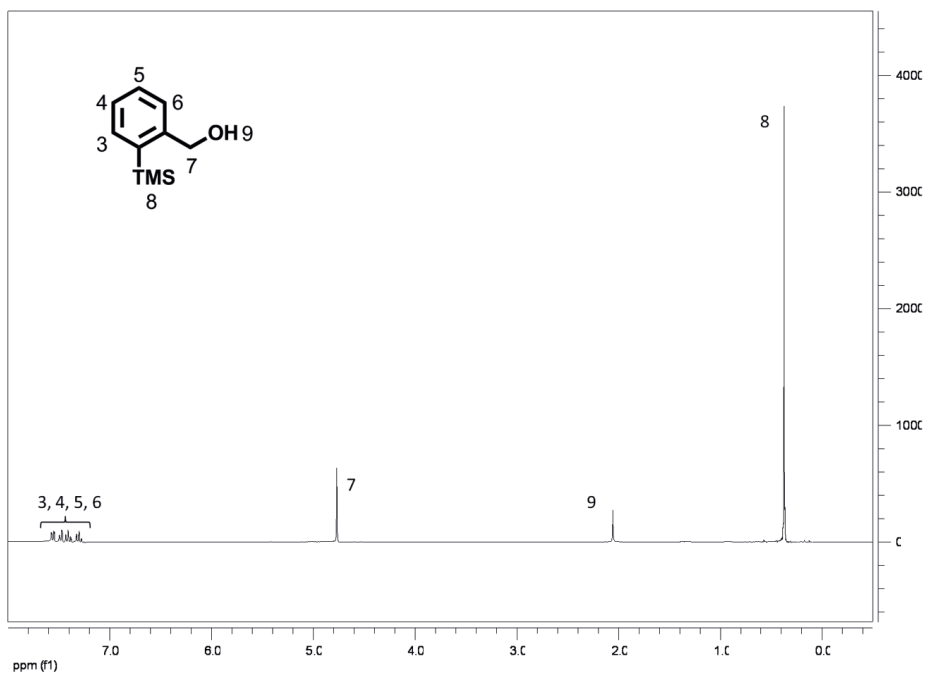
Mads H. Rønneest, Felix Nissen, Thomas Ostenfeld Larsen, Walter Mier, Mads H. Clausen *

Supporting Information

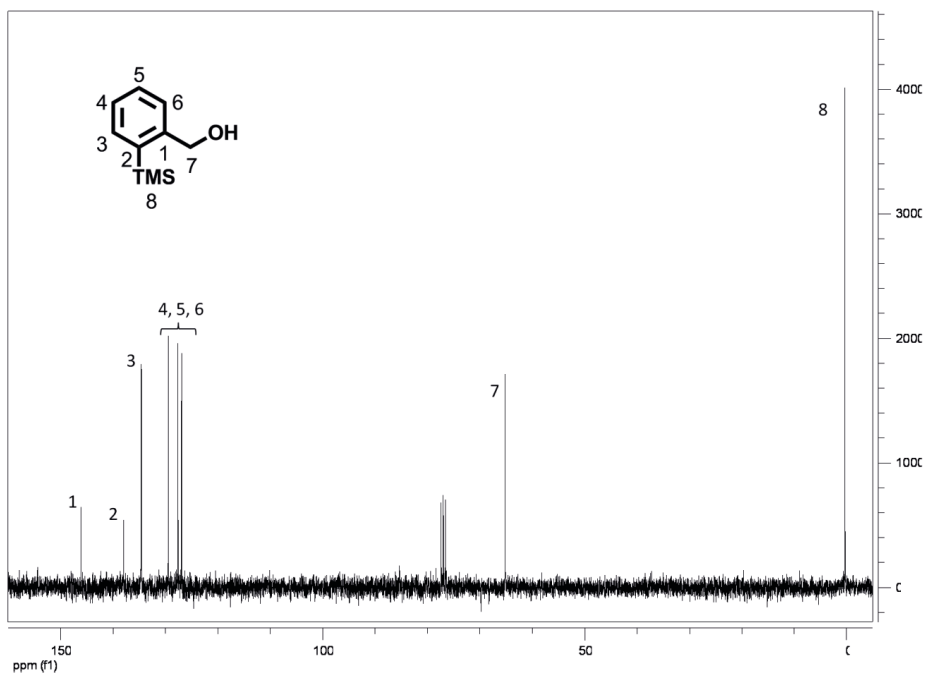
S2-S10 NMR spectra of **3**, **4**, **5**, **7**, **8**, **9**, **10**, **11** and **12**.

S11 UPLC-DAD chromatograms of compounds **7**, **8**, **9**, **10**, **11** and **12**.

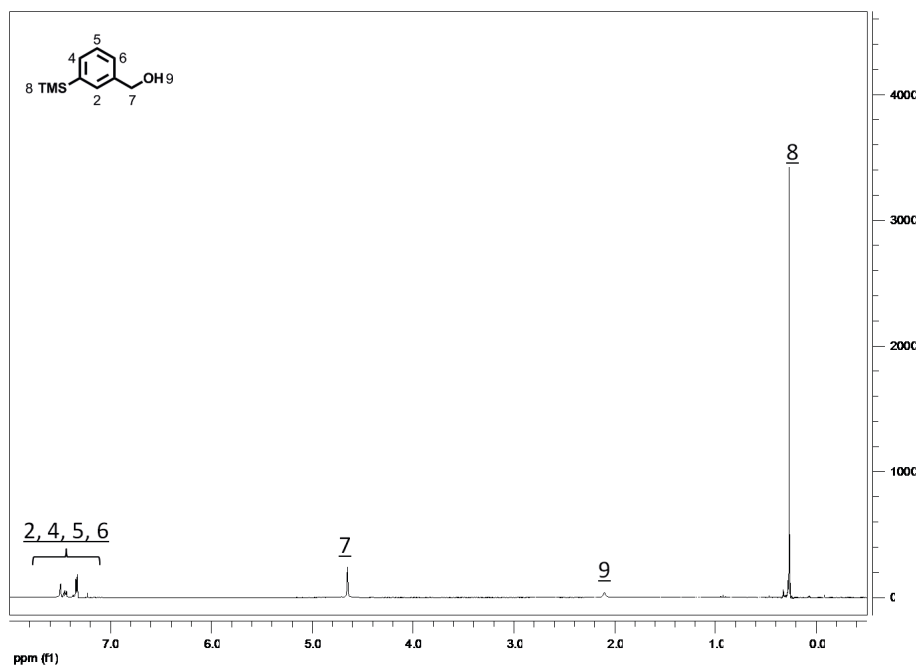
^1H NMR (300 MHz, CDCl_3) **3**



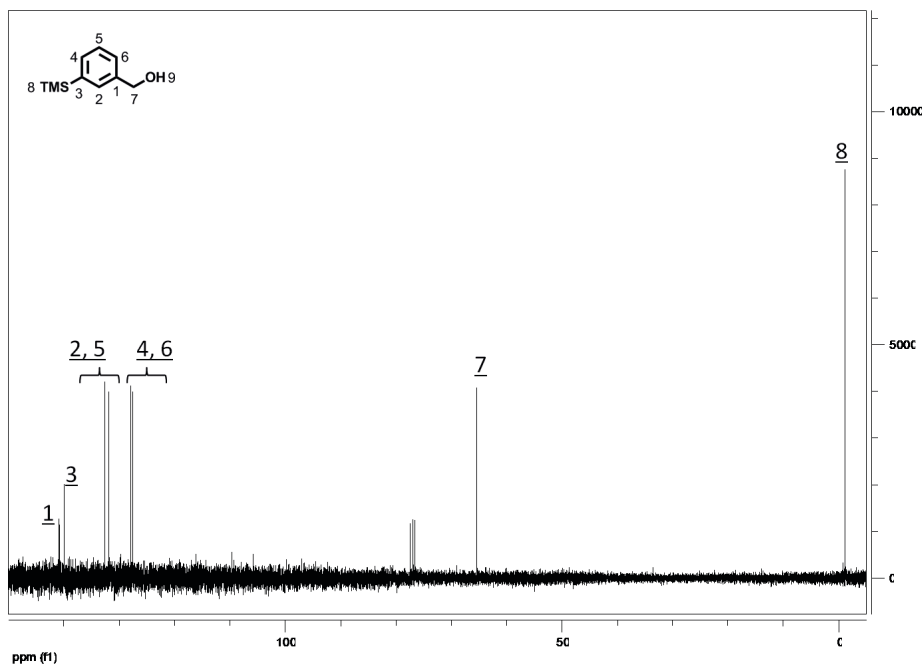
^{13}C NMR (75 MHz, CDCl_3) **3**



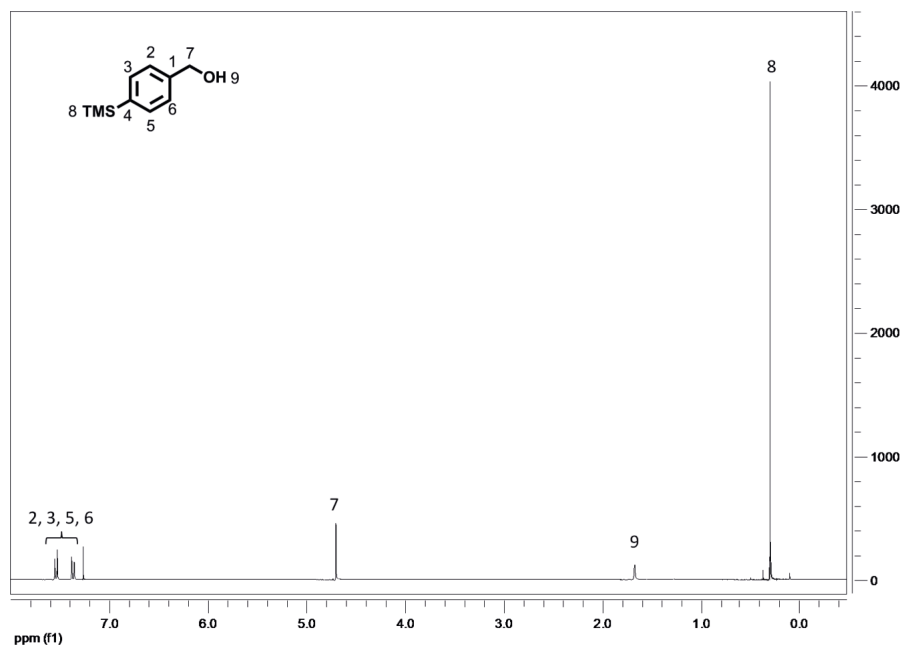
^1H NMR (300 MHz, CDCl_3) **4**



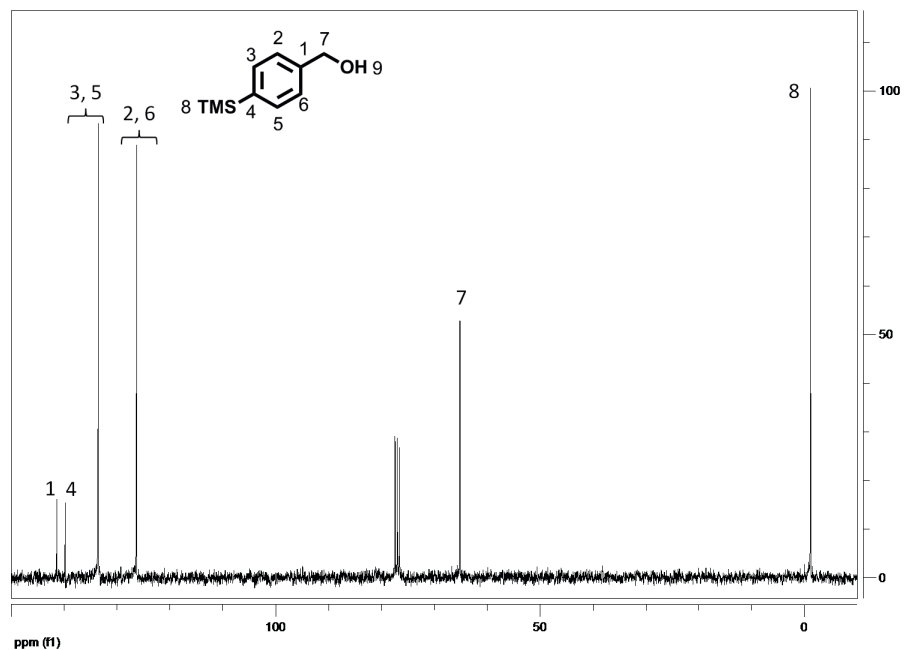
^{13}C NMR (75 MHz, CDCl_3) **4**

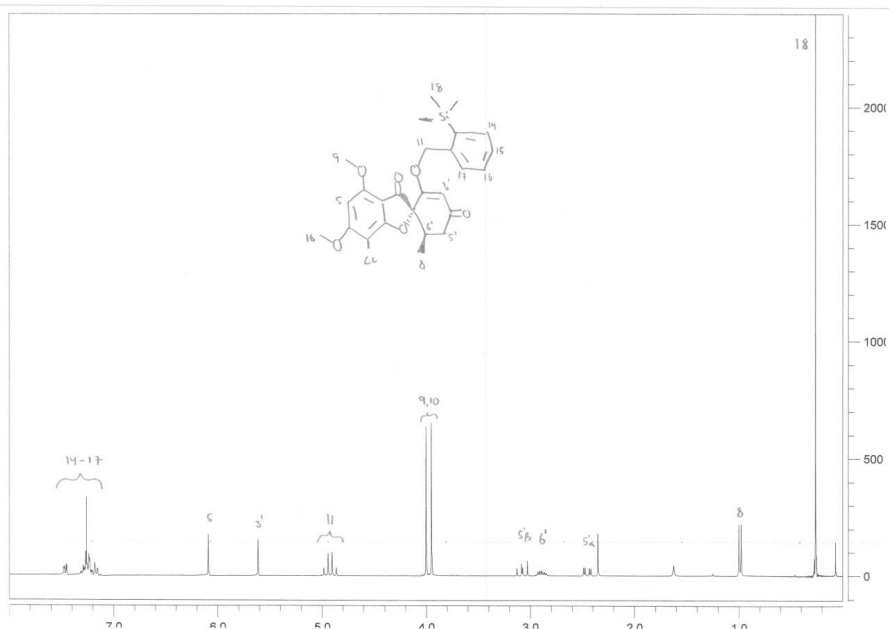
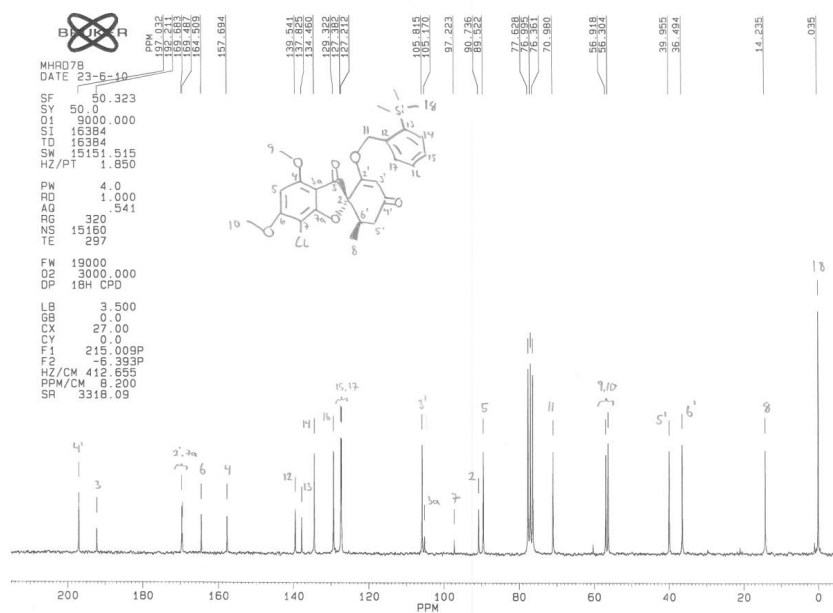


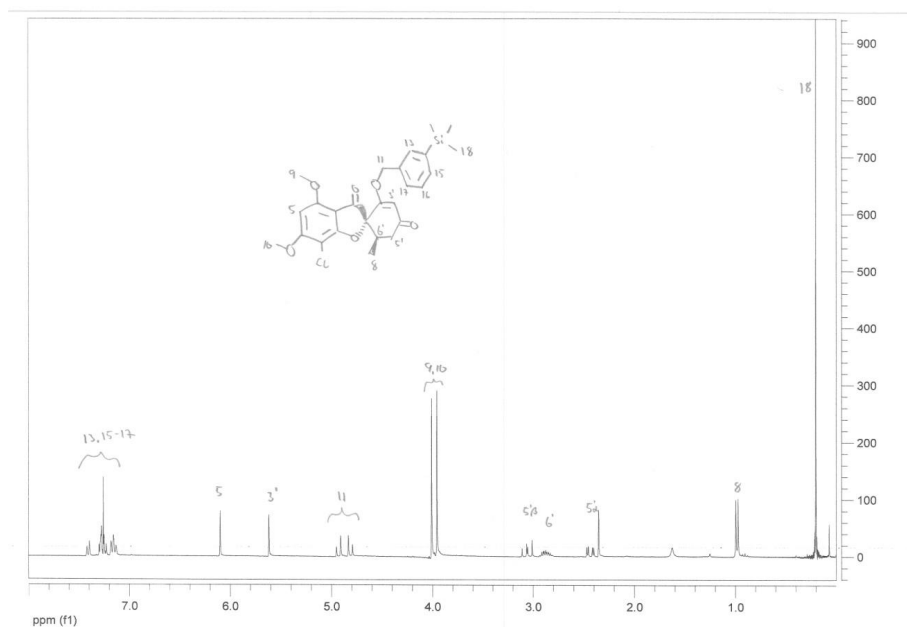
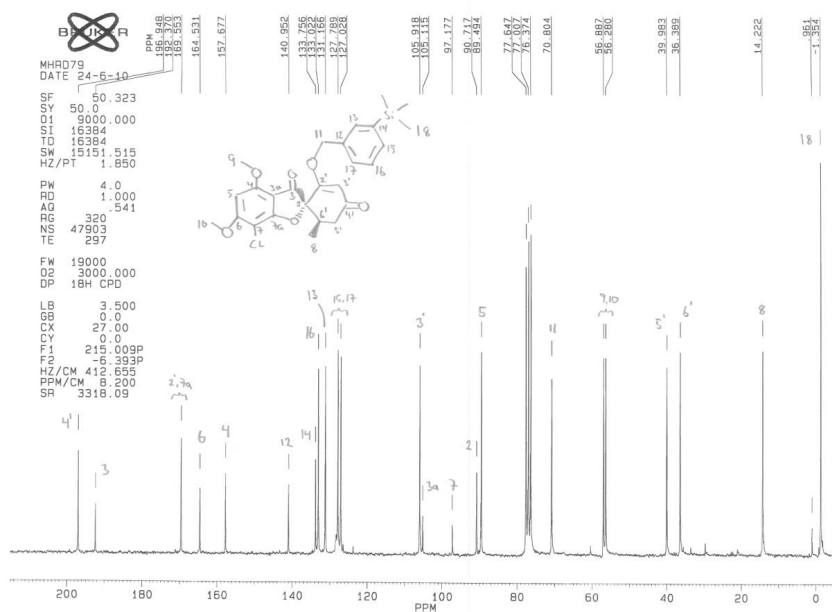
^1H NMR (300 MHz, CDCl_3) **5**

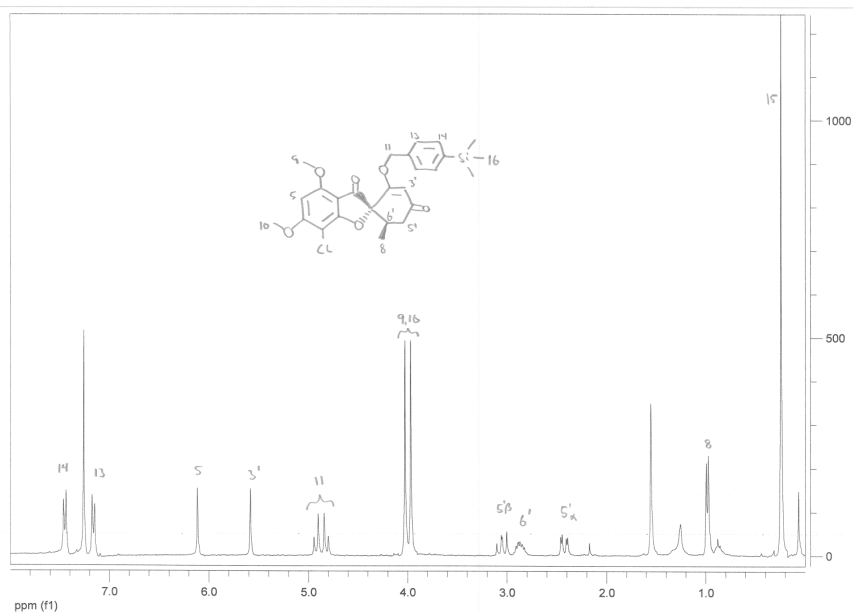
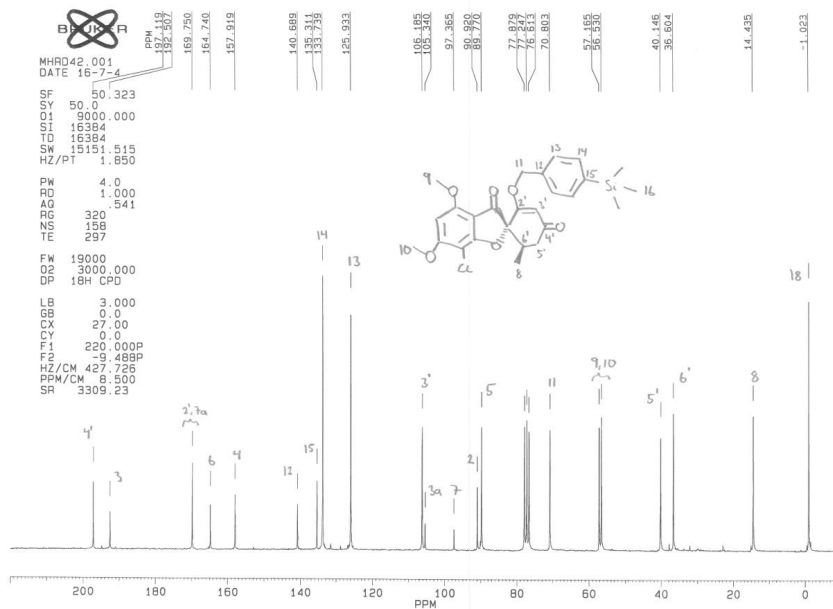


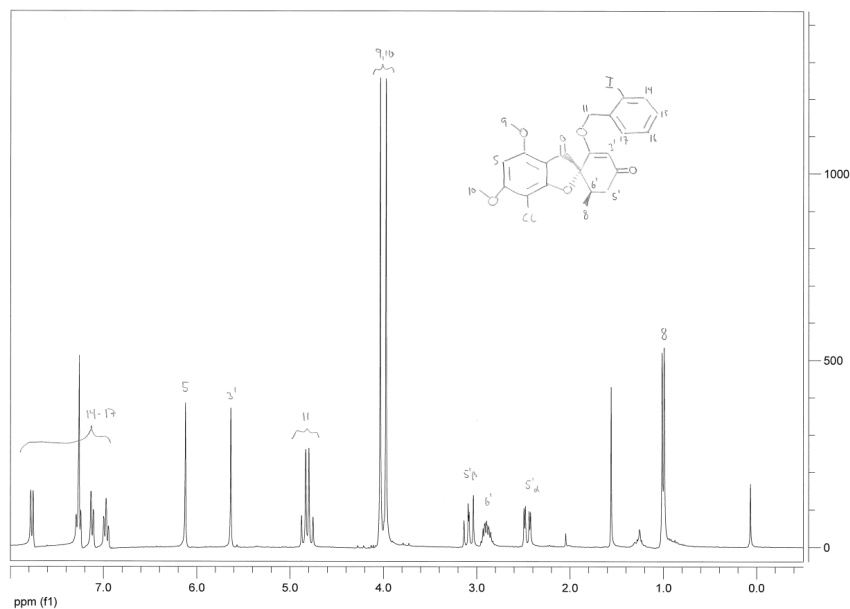
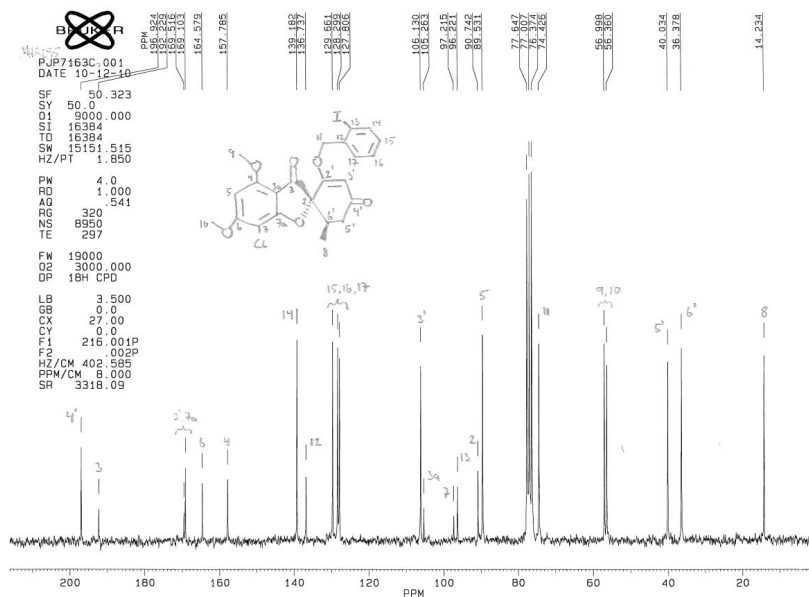
^{13}C NMR (75 MHz, CDCl_3) **5**

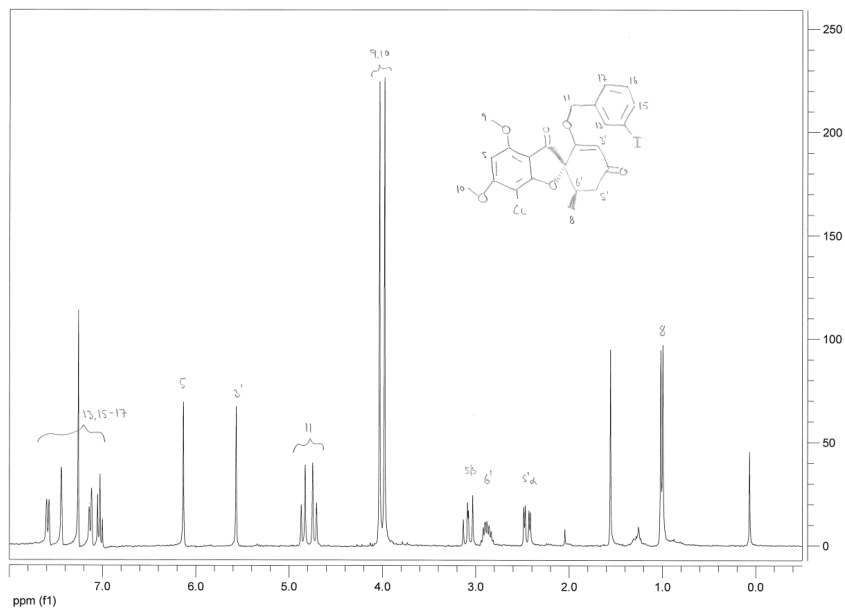
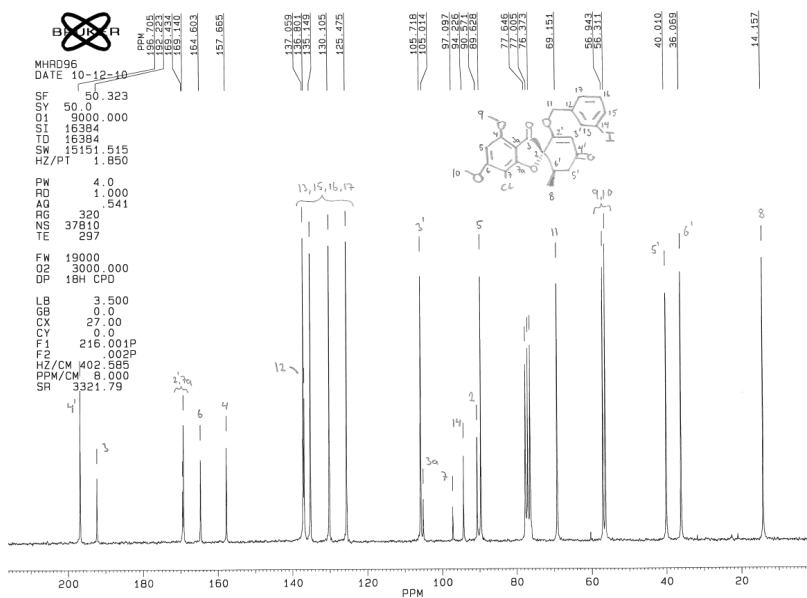


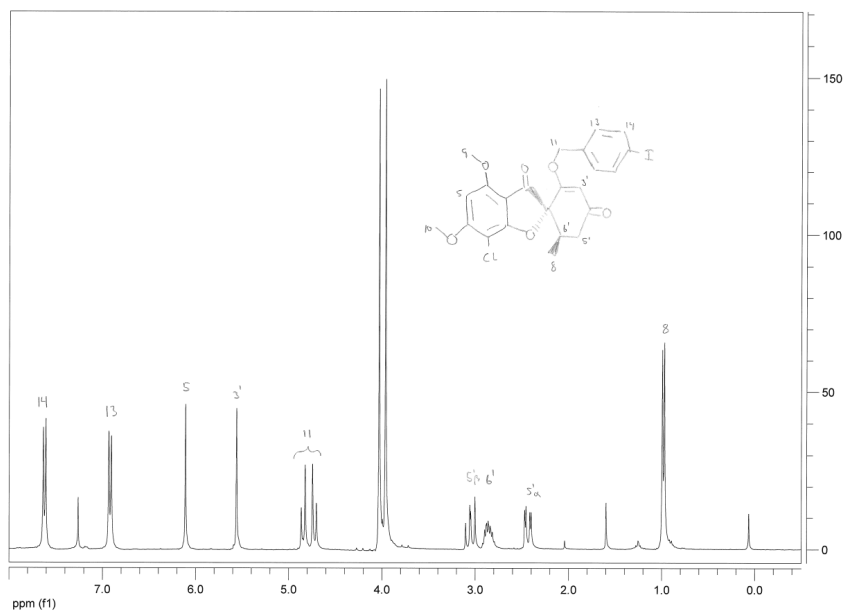
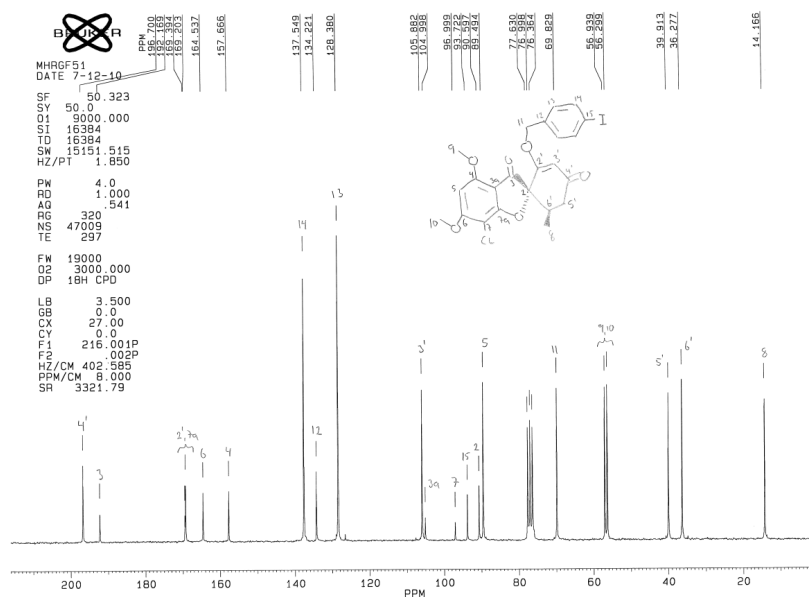
^1H NMR (300 MHz, CDCl_3) 7 ^{13}C NMR (50 MHz, CDCl_3) 7

¹H NMR (300 MHz, CDCl₃) **8**¹³C NMR (50 MHz, CDCl₃) **8**

¹H NMR (300 MHz, CDCl₃) **9**¹³C NMR (50 MHz, CDCl₃) **9**

^1H NMR (300 MHz, CDCl_3) **10** ^{13}C NMR (50 MHz, CDCl_3) **10** ^1H NMR (300 MHz, CDCl_3) **11**

 ^{13}C NMR (50 MHz, CDCl_3) 11 ^1H NMR (300 MHz, CDCl_3) 12

¹³C NMR (50 MHz, CDCl₃) 12

Supporting Information - Chapter 7

(+)-Geodin from *Aspergillus terreus*

Mads H. Rønneest, Morten T. Nielsen, Blanka Leber, Uffe H. Mortensen, Alwin Krämer, Mads H. Clausen,
Thomas O. Larsen, Pernille Harris*

Supplementary data

S2	Experimental
S3-S4	1D NMR spectra of 1 and UPLC-DAD chromatogram
S5	References

Experimental

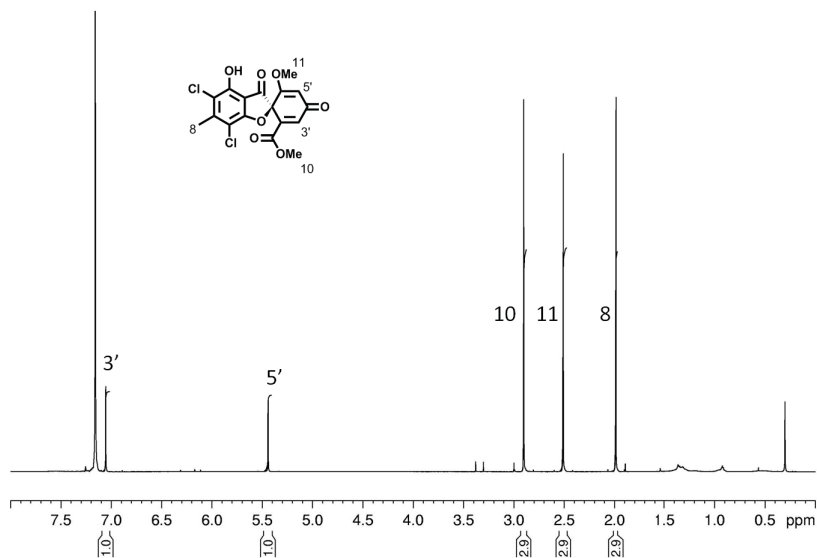
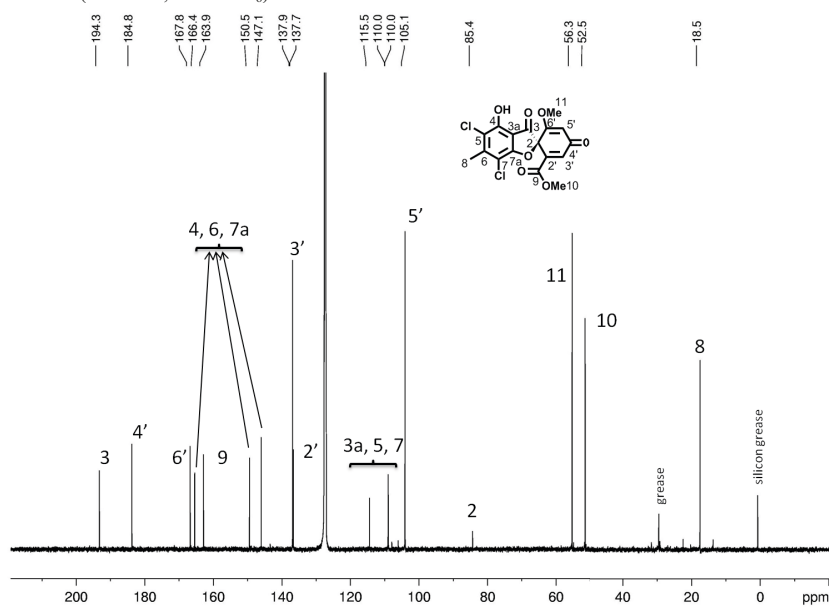
^1H NMR spectra were recorded using either a Varian Unity Inova 500 MHz or a Bruker Avance 800 MHz spectrometer. ^{13}C NMR spectrum were recorded using a Bruker Avance 800 MHz spectrometer. Chemical shifts were measured in ppm and coupling constants in Hz. When benzene- d_6 was used as solvent the residual peak was used as internal reference at 7.15 for ^1H NMR and δ 128.0 for ^{13}C NMR spectra. For DMSO- d_6 the value were δ 2.50 for ^1H NMR. The IR spectrum was recorded using a Bruker Alpha ATR and measured in cm^{-1} . The melting point is uncorrected. High-resolution LC-DAD-MS was performed on an Agilent 1100 system equipped with a photodiode array detector (DAD) and coupled to a LCT orthogonal time-of-flight mass spectrometer (Waters-Micromass, Manchester, UK) with a Z-spray electrospray ionisation (ESI) source and a LockSpray probe (M+H 556.2771) and controlled by MassLynx 4.0 software. LC-MS calibration from m/z 100-900 was done with a PEG mixture. Standard separation involved a LUNA 2 column with an acetonitrile (50 ppm formic acid) in water gradient starting from 15% to 100% over 25 minutes with a flow rate of 0.3 mL/min. Purity was assessed by UPLC-DAD on a Dionex ultimate 3000 system. The column used was a Kinetex (150×2.10 mm, $2.6 \mu\text{m}$, C-18) at 357°K with a flow of 0.8 mL. The following gradient was used:

Time (min.)	MeCN %
0	15
0.5	25
6.0	65
7.0	100
8.5	100
9.0	15
12.0	15

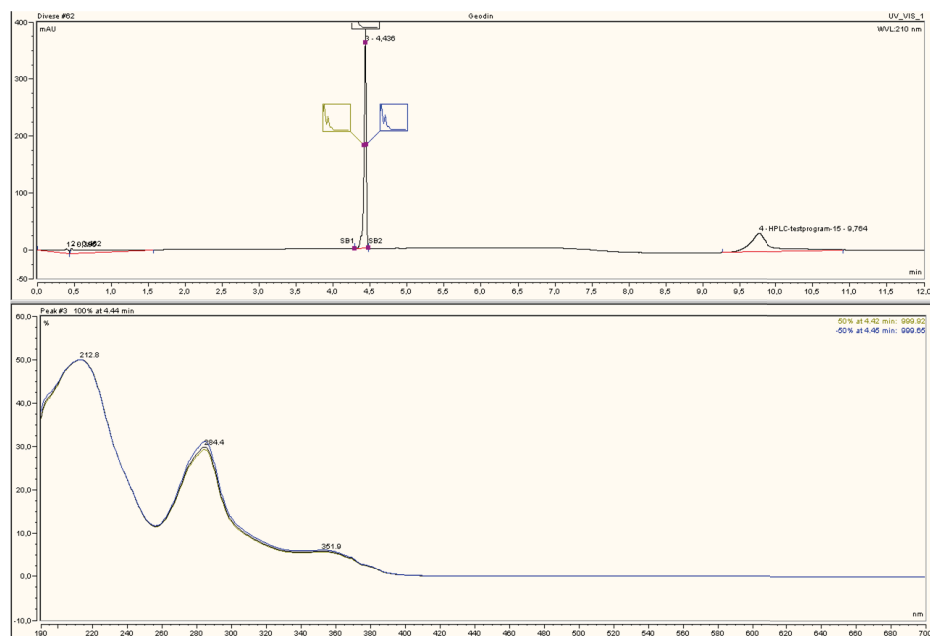
A. terreus (IBT 28226, culture collection at center for microbial biotechnology (Lyngby, Denmark)) was cultured on 50 plates of yeast extract sucrose agar at 298°K for 7 days and extracted with ethyl acetate (2L) then concentrated to afford 1.2 g raw extract. The raw extract was dissolved in 10% H_2O in MeOH (50 mL) and the aqueous phase was extracted with heptane (50 mL). The water content was increased to 50% by adding 40 mL H_2O and shaken with CH_2Cl_2 (90 mL). The CH_2Cl_2 phase was concentrated (0.86 g) and further purification was performed on a Luna HPLC column (250×10 mm, $5 \mu\text{m}$, C-18) using 5 mL/min $\text{H}_2\text{O}/\text{CH}_3\text{CN}$ (isocratic run at 50/50 for 15 minutes) as the mobile phase to yield **1** (11.6 mg as yellow oil). Geodin (**1**) was crystallized from EtOAc/heptane to afford yellow crystals.

(*R*)-Methyl 5,7-dichloro-4-hydroxy-6'-methoxy-6-methyl-3,4'-dioxo-spiro[benzofuran-2,1'-cyclohexa-2',5'-diene]-2'-carboxylate **1** (geodin)

m.p.: $527\text{--}529^\circ\text{K}$. (in agreement with litt. (Raistrick & Smith, 1936)). IR(neat): 3396, 1724, 1659, 1610, 1461, 1440; ^1H NMR (800 MHz, benzene- d_6): δ 7.04 (1H, d, $J = 1.0$ Hz), 5.43 (1H, d, $J = 1.0$ Hz), 2.91 (3H, s), 2.53 (3H, s), 2.00 (3H, s); ^{13}C NMR (200 MHz, benzene- d_6): δ 194.3, 184.8, 167.8, 166.4, 163.9, 150.4, 147.1, 137.9, 137.7, 115.5, 110.0, 110.0, 105.1, 85.4, 56.3, 52.5, 18.5; ^1H NMR (800 MHz, CDCl_3): δ 7.14 (1H, d, $J = 1.5$ Hz), 5.82 (1H, d, $J = 1.5$ Hz), 3.74 (3H, s), 3.70 (3H, s), 2.58 (3H, s) (in agreement with litt. (Sato *et al.*, 2005)); ^1H NMR (500 MHz, DMSO- d_6): δ 7.05 (1H, d, $J = 1.0$ Hz), 6.05 (1H, d, $J = 1.0$ Hz), 3.72 (3H, s), 3.68 (3H, s), 2.52 (3H, s); HRMS (ESI+) calcd for $\text{M} + \text{H} [\text{C}_{17}\text{H}_{13}\text{Cl}_2\text{O}_7]^+$ 399.0038, found 399.0041. $[\alpha]_{\text{D}}^{21} +129^\circ$ ($c = 1.16$, CHCl_3) (in agreement with litt. (Raistrick & Smith, 1936)).

¹H NMR (800 MHz, benzene-*d*₆)¹³C NMR (200 MHz, benzene-*d*₆)

UPLC-DAD chromatogram and UV spectrum



References

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Center for Microbial Biotechnology
Department of Systems Biology
Technical University of Denmark

Building 223
DK-2800 Kgs. Lyngby
Denmark

Phone: +45 4525 2525
Fax: +45 4588 4148

www.cmb.dtu.dk

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